PROFILING THE IMMUNE RESPONSE IN EARLY INFLAMMATORY BOWEL DISEASE

Carolijn Smids

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CONTENTS

1

General introduction and outline of the thesis

THE INGENIOUS INTESTINAL MUCOSA: DISRUPTED IN INFLAMMATORY BOWEL DISEASE

The human gut must perform a constant balancing act, as it needs to transport nutrients, vitamins and ions from the luminal side to different tissues, while on the other hand it keeping out toxic and harmful pathogens. Furthermore, the mucosal immune system needs to tolerate microorganisms that are not pathogenic, whilst reacting robustly against pathogenic microorganisms. Underneath the mucus layer hides an interplay of numerous cells with highly specialized functions to execute these tasks. Surprisingly, these processes work well in most people, but dysregulation lies in wait, as is seen in patients with an inflammatory bowel disease (IBD) [1]. Patients with IBD suffer from a chronic inflammation of the gastrointestinal tract which causes a lifelong, debilitating disease.

INFLAMMATORY BOWEL DISEASE: EPIDEMIOLOGICAL AND CLINICAL ASPECTS

IBD encompasses Crohn's disease (CD), ulcerative colitis (UC) and IBD unclassified (IBDU). The disease affects about one in two hundred people in high-income countries, and incidence is rapidly increasing [2,3]. In Western Europe, UC emergence coincided with the industrial revolution in the 1800s, and its incidence slowly increased in the early 1900s [3]. From 1950 onwards, there was a rapid expansion of the incidence of IBD in Westernized countries, explained by factors like increased awareness, improved medical access and environmental exposures associated with Westernization of the population (e.g. smoking, changing diet). Since 1990, IBD incidence has stabilised in the Western world [4]. However, globalization at the end of the 20th century, with Westernization of parts of Asia, the Middle East, and South Africa, was again paralleled by an explosive increase in IBD incidence and prevalence. Recent data on a (South-Limburg) cohort of the Dutch population revealed an incidence of IBD in 2010 of 40.36 per 100.000 people, with CD and UC incidence of respectively 17.49 and 21.47 per 100,000, with a nationwide IBD prevalence estimated to be 613 per 100,000 inhabitants (approximately 80,627 people) [5].

Patients with IBD suffer from a chronic disease characterised by unpredictable episodes of relapse and remission, inflicting symptoms like diarrhoea, blood in their stool, urgency, abdominal pain, weight loss, fever and fatigue. The diagnosis IBD is usually established between the ages of 20-40 and based on a combination of clinical, endoscopic, histological and radiological criteria according to internationally accepted guidelines [6-9]. IBDU is diagnosed in 10-15% of patients, when there is diagnostic uncertainty, and in the absence of surgical resection material of ileum or colon [10,11]. The disease characteristics of CD and UC are described in table 1. Treatment consists primarily of anti-inflammatory (immunosuppressive) drugs and surgery when there is lack of response to medical treatment. The IBD drug regime currently

consists of: 5-aminosalicylic acid, corticosteroids, thiopurines, methotrexate, biological treatment targeting cytokines (anti-TNF/anti-IL12/23 p40) and anti-integrin treatment targeting leukocyte trafficking (anti-α4β7), with several emerging treatment options nearly entering the stage.

Table 1. Characteristics of Crohn's disease and ulcerative colitis

	Crohn's disease	ulcerative colitis
Disease location	Mouth to anus involving all gut layers (mucosa to serosa)	Colon, only involving mucosa
Endoscopy findings	Segmental distribution, rectum frequently spared, aphthous ulcers, deep fissuring ulcers, cobblestoned appearance bowel wall, perianal disease, strictures, fistulas	Diffuse and continuous erythema from the rectum upwards, friability, loss of vascular pattern
Radiology	Small bowel abnormalities, ileal abnormalities, skip areas, strictures, fistulas	Normal small bowel, (pan)colitis, no skip areas dilatation of colon in toxic megacolon
Histology	Pathognomic non-caseating granulomas, patchy cryptitis, crypt abscesses, ileitis, inflammation extends through the mucosa and muscle of the bowel	Cryptitis, crypt abscesses or architectural distortion, granulomas absent, inflammation confined to mucosa
Clinical findings	Abdominal pain, diarrhoea, rectal bleeding, weight loss, anorexia, fever, fatigue, extra-intestinal manifestations	Bloody diarrhoea, abdominal pain, weight loss, fever, fatigue extra-intestinal manifestations

Uncomplicated mucosal inflammation is present in 70-80% of CD patients at diagnosis, whereas 20-30% already have complicated disease behaviour with penetrating or stricturing lesions, leading to fistulae, abscesses or obstruction[12-14]. The latter percentage extends up to 50% after 20 years in a population-based cohort and 88% in a referral-based population [12-14]. Half of the CD patients require surgical resection during the first 10 years of disease, but this risk is decreasing over time in an era of changing medical therapy [15]. Frequently described clinical determinants at presentation of CD associated with a complicated disease course are younger age (<40), perianal or stricturing disease, involvement of the upper gastrointestinal tract, penetrating disease and smoking [16,17]. However, the clinical spectrum of CD is very heterogeneous and cannot be predicted precisely.

UC also has great clinical heterogeneity between patients. Colectomy is a sign of a complicated disease course in UC and is required in approximately 10% of patients in the first 10 years of disease [18]. Extensive colitis at presentation, as well as severe systemic symptoms, were found to be predictors of colectomy [19,20]. Proximal extension of disease (from proctitis/left-sided colitis to extensive colitis) occurs in 14-28%, and is associated with a greater risk of undergoing colectomy than patients with extensive disease at diagnosis [19]. Cumulative incidence of a

colorectal carcinoma in UC patients was initially reported to be 0.4% after 10 disease years, and 1.1% after 20 years of follow-up [21]. These rates are declining over time, probably related to improved medical treatment and better patient care [22].

Patients with IBD suffer from disease symptoms as well as from long term complications, both caused by the disease as by different medical and surgical treatments. Physical and psychosocial functioning may be impaired and future perspective uncertain, which will have consequences for general quality of life, personal relations and professional career. Furthermore, IBD leads to substantial financial costs, with annual costs of per-patient-care of €7.835 for CD and €3.600 for UC patients in the Netherlands [23].

Currently, both our ability to predict disease behaviour in individual patients, as well as our insight in response of patients to different medications is limited. Historically, treatment is based on generic protocols, lacking individual profiles. Therapy is based on disease activity or the development of complications. Adequate monitoring is therefore essential to make timely decisions. As the population of IBD patients represents a heterogeneous group with great diversity in clinical presentation, severity, progression and response to therapy, therapeutic requirements vary considerably and an optimal personalized medicine approach is warranted [24-26].

Another important argument for the necessity of personalized medicine in IBD is the frequent discordance between symptoms and the presence or severity of mucosal inflammation, more so in CD patients than in UC. On one hand, patients present themselves with symptoms without actual mucosal inflammation, which can lead to overtreatment. On the other hand, patients with active mucosal inflammation may not experience any symptoms, leading to undertreatment and potentially progression to complicated disease. This renewed focus on mucosal inflammation urges the identification of biomarkers of inflammation to implement personalized treatment. Invasive and costly procedures such as ileocolonoscopy and cross-sectional imaging inform the clinician about the inflammatory state of disease. The use of laboratory tests in monitoring disease activity and predicting complicated disease or treatment response would be more convenient, non-invasive, standardized, rapid, reproducible and inexpensive. Until now, the most extensively studied non-invasive biomarkers are serum CRP levels and faecal calprotectin levels, but these merely reflect (potential) disease activity rather than predicting disease course [27]. On top of the list of key research priorities regarding optimization of IBD treatment strategy are selection of the right patient group and phase of disease and identification of markers for patient stratification regarding disease course [28].

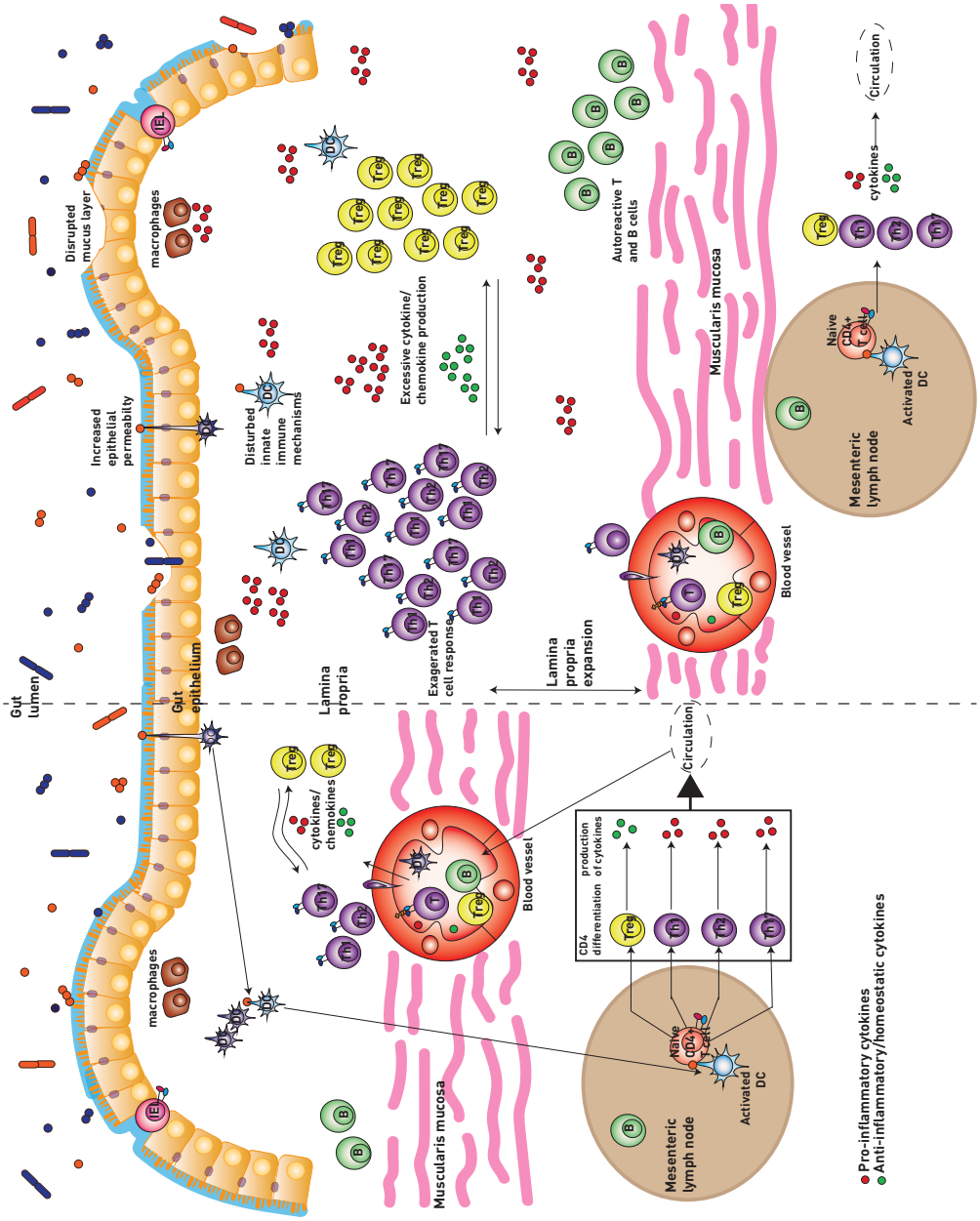
INFLAMMATORY BOWEL DISEASE: PATHOGENESIS

As in most autoimmune diseases, the aetiology of IBD is explained by multifactorial contributions requiring interactions between environmental factors, a disturbance of the gut microbiome, genetic predisposition and dysregulation of the immune system [29,30]. Environmental

determinants include external factors like cigarette smoking, antibiotic use in childhood, and changing diet [3]. Even though some risk factors have been identified, it has not yet been shown that modifying exposure actually prevents IBD development. Imbalance of the composition of the gut microbiome, the ecosystem of microorganisms residing in mucosal surfaces (internal environment), has also been proposed as pathogenic factor [31]. However, identification of an altered microbiome is complicated by intra- and inter-individual variation, even present in healthy individuals [32]. Much of the current literature on microbiome in IBD concerns patients with established disease and therefore under immune modulating treatment, which must be considered when interpreting results [33]. These results underscore impaired managing of microbial antigens by the intestinal immune system in IBD, but no single species has been proven to be exclusively causative [34,35]. Currently, no predictive statements can be made based on the microbiome of IBD patients. Genome-wide association studies in IBD patients start to shed more light on associated risk alleles [36]. Since nucleotide oligomerisation domain 2 was identified as the first susceptibility gene for CD in 2001 [37], over 200 susceptibility loci for IBD have been found. However, the largest performed genotype-phenotype study in IBD found only three loci associated with phenotypes of disease location in IBD and most individuals with IBD-associated genetic risk variants remain healthy. This large study did not find any association with disease behaviour. Genetic predictors may therefore only explain a small fraction of phenotypic variance, and they cannot be used as a predictor in individual patients [38]. The inflammatory processes in IBD are executed primarily by immune cells. Therefore, this factor has dominated the investigation of IBD pathogenesis, with focus on antibody responses, T cells, cytokines as well as innate immunity. As this thesis focuses on the disrupted immunological processes in IBD, this will be described in to more detail separately. The complex of interacting aetiological aspects of IBD may be responsible for the clinical heterogeneity of the disease.

Figure 1. The intestinal immune system in health and inflammatory bowel disease.

In healthy state (left panel) the lamina propria contains a diverse array of immune cells which maintain a homeostatic balance (innate cells like macrophages and dendritic cells (DCs) as well as adaptive cells including B lymphocytes and T lymphocytes like regulatory T lymphocytes (Treg), and T helper lymphocytes (Th1, Th2, Th17)), and both pro- and anti-inflammatory cytokines/chemokines. DCs can be activated by intestinal bacteria, they present antigens to naïve $CD4^+$ T cells in secondary lymphoid organs like the mesenteric lymph node, where the phenotype of the DC as well as the cytokine milieu modulate CD4 subgroup differentiation and its characteristic cytokine profile. These activated T cells then circulate to the lamina propria to carry out their effector function. In patients with inflammatory bowel disease patients (right panel) the homeostatic balance is disturbed at all levels of defence with a disrupted mucus layer, increased epithelial permeability, disturbed innate immune mechanisms, exaggerated T cell responses, excessive cytokine/chemokine production and the production of autoreactive T and B cells inducing marked expansion of the lamina propria with the increased number of immune cells.


Gut lumen
Gut epithelium
Lamina propria
Lamina propria expansion
Disrupted mucus layer
Increased epithelial permeability
Disturbed innate immune mechanisms
Excessive cytokine/ chemokine production
Exagerated T cell response
Autoreactive T and B cells
Muscularis mucosa
Blood vessel
Circulation
cytokines
Mesenteric lymph node
Naive CD4+ T cells
Activated DC
CD4 differentiation
production of cytokines
Pro-inflammatory cytokines
Anti-inflammatory/homeostatic cytokines

HUMAN INTESTINAL IMMUNE HOMEOSTASIS AND ITS DISRUPTION IN IBD PATIENTS

In healthy individuals, the intestinal immune system contains a diverse spectrum of immune cells and cytokines to maintain a homeostatic balance [29,39,40]. This includes pro-inflammatory mediators to limit excessive entry of microorganisms in a defence against pathogens, and anti-inflammatory mediators to down-regulate immune responses. Numerous microorganisms inhabit the gut lumen; pathogens and commensals, the latter contributing to the maintenance of homeostasis. The first line of defence against pathogenic microorganisms in the gut is the epithelial barrier; a single layer of enterocytes and specialized epithelial cells held together by tight junctions and covered on the luminal side by a thick mucus layer embedded with commensal microbes (figure 1). Besides being a physical barrier, by preventing the penetration of macromolecules and bacteria, epithelial cells can also secret bactericidal agents called defensins.

Beneath the epithelial layer lies the loose connective tissue of the lamina propria. Cells from the innate immune system can be found here, including dendritic cells (DCs), macrophages and neutrophils, which can sense danger signals leading to their production of cytokines and antimicrobial peptides. Cytokines are involved in the regulation, development and activation of immune cells to mediate the inflammatory response. After antigen recognition, DCs can be activated to mature and perform its function as antigen presenting cell activating the adaptive immune response. Adaptive immune cells, also present in the lamina propria, include T lymphocytes and B lymphocytes. Directed by the DCs, they can either act in an inflammatory way to fight off pathogens or in a regulatory fashion to maintain tolerance to self-antigens. This delicate equilibrium is not always perfect, as autoimmune diseases are characterized by an excess of T and B cell reactivity. T and B cells, as well as monocytes and DCs, enter the periphery by homing to secondary lymphoid organs, awaiting antigen activation. In the gut these organs are collectively called gut associated lymphoid tissue (including Peyer's patches and mesenteric lymph nodes). Activated T lymphocytes circulate to the intestinal lamina propria, through the intestinal blood vessels, where they can carry out their regulatory and effector functions.

In IBD there is an inappropriate response of the intestinal immune system to luminal antigens and the homeostatic balance is disturbed at all levels of defence, showing leaky epithelial barriers, disturbed innate immune mechanisms, excessive cytokine/chemokine production, incorrect recognition of commensal antigens by DCs and disturbed clearance of autoreactive T and B cell populations (figure 1). Circulating antimicrobial antibodies in IBD patients indicate B cell reactivity. An exaggerated T lymphocyte response is often implicated as key mediator in the development and maintenance of IBD [41]. The main focus of research on T lymphocytes in IBD has been directed on the activated $CD4^+$ T effector lymphocytes (Th1, Th2, and Th17) and regulatory T cells (Tregs), and was largely based on cytokines detected in the gut mucosa

of mice models and longstanding treated IBD patients [42-45]. Previously, our group studied T lymphocytes in the inflamed gut mucosa of IBD patients at diagnosis, and identified patient subgroups according to different stages of T cell maturation [46]. These different subgroups might have predictive value for treatment response.

AIMS OF THIS THESIS

Studies in adult IBD patients on immunological responses are mainly cross-sectional, often single-time-point studies, in longstanding IBD, after complications have already occurred and under immunosuppressive medication. Baseline results at the onset of disease are scarcely available. We believe that there is a small window of opportunity to study early inflammatory processes and candidate biomarkers in IBD patients at initial diagnosis, just before treatment is initiated. Therefore, at Crohn's and Colitis Centre Rijnstate, we prospectively enrolled IBD patients solely at the moment of diagnosis, all with endoscopic confirmed inflammation, and studied intestinal biopsy specimens as well as venous blood samples drawn directly after endoscopic evaluation. Patients were longitudinally followed-up, and a second venous blood sample was collected at an arbitrary visit to our hospital, as well as a second analysis of biopsy specimens when endoscopy was performed according to standard care, to study stability and behaviour of different immunological profiles over time. We identified immunological profiles regarding mucosal T lymphocyte subsets, serum antibodies and other serum analytes (cytokines/chemokines) at presentation and assessed changes over time (from diagnosis to follow-up) as well as relations to disease activity, severity, course and response to therapy. This may enable individualization of patient care.

OUTLINE

Chapter 1 introduced the background information and framework of this thesis. A review on the literature on maturation and migration of T lymphocytes in and around the gut mucosa in homeostasis and IBD can be found in **chapter 2**. We hypothesize on the potential role of tertiary lymphoid organs (TLOs) in the gut mucosa to facilitate the influx of certain T lymphocyte subsets in IBD patients through high endothelial venules (HEVs) in **chapter 3**. The presence of these HEVs and TLOs in the inflamed gut mucosa was evaluated in newly diagnosed, untreated IBD patients; in relation to intestinal T lymphocytes. In **chapter 4** we investigate the behaviour of mucosal T lymphocyte subsets in IBD patients at diagnosis and over time, and related them to disease activity and -outcomes. We explore the physiological, and potential pathological, roles of (mucosal) immune cells expressing integrin αEβ7 (mainly T lymphocytes) in health and IBD (**chapter 5**) as it is targeted by anti-β7 treatment. We question the pathological role of $\alpha E\beta 7^{+}$ T lymphocytes in IBD patients in **chapter 5a** and provide a detailed background overview on this topic in **chapter 5b**. Serum antibodies in newly diagnosed IBD patients, as well as their presence over time, were assessed in **chapter 6** in relation to disease phenotype and course. We identified candidate serum markers in CD patients at presentation and during their follow-up associated with disease activity and –course in **chapter 7.** This thesis is completed in **chapter 8** with a summary of the results and a discussion on the future perspectives on mucosal T lymphocyte subsets and other potential markers in IBD patients.

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2

On naivety of T cells in Inflammatory Bowel Disease: A review

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ABSTRACT

Little is known about different phases of T cell maturation in gut mucosa. Based on current knowledge about the migratory pathways of naïve and memory T cells, it is believed that access to peripheral, non-lymphoid tissues is restricted to memory T cells. Surprisingly, there is increasing evidence of high numbers of naïve T cells in the chronically inflamed gut tissue of patients with inflammatory bowel disease. This could partially be explained by new formation of ectopic lymphoid organs. Ongoing recruitment of naïve T cells at inflammatory sites might play a role in the immunopathogenesis of inflammatory bowel disease.

INTRODUCTION

Inflammatory bowel diseases (IBD) are believed to result from aberrant inflammatory responses to the intestinal bacterial antigens in a genetically susceptible individual. Available evidence argues an important role for the adaptive immune system in IBD. Most research has focused on increased migration and proliferation of activated or memory T cells (Th1, Th2, Th17 and regulatory T cells) in the inflamed gut mucosa [1,2]. As naïve T cells (T_N) are normally thought to be excluded from inflamed tissue, the maturation process at extra-lymphoid effector sites like the inflamed gut mucosa in IBD, has received less attention. However, T_N are the initiators of the inflammatory cascade of activated T cell expansion and differentiation, and may therefore play a central role in inflammatory processes.

Lymphoid progenitors originating from bone marrow stem cells undergo maturation and selection in the thymus to become functional $CD4^+CD8^-$ or $CD4^-CD8^+$ (single positive) T lymphocytes. In this thymic selection process, T cells that can recognize peptide in the context of self-MHC molecules are positively selected whereas auto-reactive T cells are deleted in the thymus [3]. Of all T cells entering the thymus, the stringent thymic selection leaves only 3% [4]. The T_N cells leaving the thymus still have to experience antigen driven post-thymic maturation before they proliferate and differentiate into memory T cells [5]. The classical view states that T_N cells exclusively recirculate between secondary lymphoid organs (SLOs, e.g. lymph nodes, Peyer's patches (PPs) and the spleen) through lymph and blood, whereas only memory T cells are able to enter non-lymphoid organs [6]. However, recent evidence has shown that T_N cells routinely traffic through non-lymphoid organs and might be activated outside the confined lymphoid complex [7-16]. These findings shed new light on the role of T_N cells in autoimmune disease.

This review summarizes the current knowledge of the involvement of T_N cells in IBD. We focus on the migration and activation of these cells in the non-lymphoid, healthy and inflamed gut.

T-CELL SUBSETS: MATURATION AND ACTIVATION

Human T cells can be subdivided into different sets, either according to their immunophenotype (expression of leukocyte differentiation markers) or to their functionality (production of chemokines). Considering phenotypic characterization, the two main categories are T_N cells, which have not yet encountered any (foreign) antigen, and memory T cells, which are antigen-experienced. The phenotypic changes during the antigen driven maturation and activation process are depicted in table 1 and figure 1.

T_N cells can be subdivided in two subsets: recent thymic emigrants (RTEs), also called 'truly naïve' and mature naïve T cells (T_{MN}) [4]. RTEs have not yet met the antigen for their specific T cell receptor (TCR) and have no proliferation history. T_{MN} cells are also 'antigen-inexperienced' but underwent proliferation in response to homeostatic survival signals [17]. RTEs can be identified

by the expression of CD45RA, CD31, CD27, CD28, CCR7, and CD62L [18] and are enriched with TCR excision circles (TRECs) [4]. TRECs are non-replicative circular excision DNA products of TCR gene rearrangement [17,19]. Post-thymic proliferation of RTEs causes diminishing of TRECs, downregulation of a set of surface markers (CD31, PTK7) generating 'proliferative-experienced' T_{MN} cells [4].

Table 1. Different maturation stages of human T cells are distinguished by various cell surface markers.

Surface markers	RTE	T_{MN}	T_{CM}	T_{EM}	T_{EMRA}
CD31	+	-	-	-	-
CD45RA	+	+	-	-	+
CCR7	+	+	+	-	±
CD62L	+	+	+	-	-
CD27	+	+	+	±	-
CD28	+	+	+	±	-
CD45RO	-	-	+	+	-

Immunophenotypical subsets of T cells: RTE = recent thymic emigrant; T_{MN} = mature naïve T cell; T_{CM} = central memory T cell; T_{EM} = effector memory T cell; T_{EMRA} = effector memory T cell re-expressing CD45RA. Surface markers: CD = cluster of differentiation; CCR7 = chemokine receptor 7, CD62L = L-selectin. Normal expression (+), moderate expression (±), no expression (-).

T cells that have encountered antigen, can immunophenotypically be subdivided into central memory T cells (T_{CM}), effector memory T cells (T_{EM}) and effector memory T cells re-expressing CD45RA (T_{EMRA}). During maturation, T_{CM} express CD45RO instead of CD45RA, but retain CCR7 and CD62L, which are important homing markers for lymphoid tissue. During further maturation, T_{EM} cells downregulate both CCR7 and CD62L and acquire new adhesion markers such as the gut-homing markers $\alpha_4\beta_7$ and CCR9 [20-22]. A subset of effector memory T cells, known as T_{EMRA} cells (re)express CD45RA and occasionally also CCR7, but remain distinguishable from T_N cells by their lack of CD27, CD28 and CD62L. When defining T_N cell subsets by immunophenotype, the use of at least two markers is recommended [18]. Different combinations have been suggested: CD45RA and CD27/CD28 [18], or CCR7 and CD27 [23]. Unfortunately, this is not yet common practice and T cells are often defined as naïve or memory solely by the expression of CD45RA/RO. This makes the distinction between T_N cells and T_{EMRA} cells practically impossible. Contamination of the T_N cell subset with T_{EMRA} cells may lead to inaccurate conclusions and hamper comparability between studies.

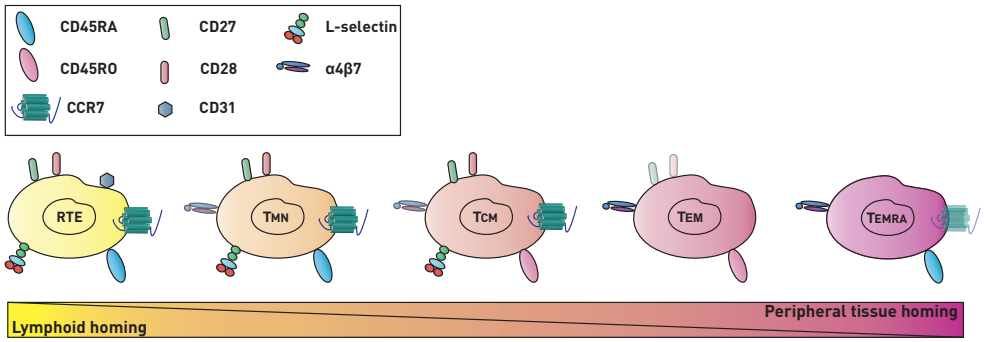

Figure 1. Human T cell maturation subsets.

Immunophenotypical subsets of T cells by expression of several cell surface markers. Intensity of colour of markers indicates level of expression. RTE = recent thymic emigrant; T_{MN} = mature naïve T cell; T_{CM} = central memory T cell; T_{EM} = effector memory T cell; T_{EMRA} = effector memory T cell re-expressing CD45RA; CCR7 = chemokine receptor 7; CD62L = L-selectin.

Migration of naïve T lymphocytes during homeostasis

Migration of immune cells is a process referred to as homing. The general belief before 1964 was that lymphocytes randomly circulate through the body. The first signs hinting otherwise were found in rats and mice [24,25], showing that certain lymphocytes only homed to the lymph node or the spleen. Thirty years later, these findings were confirmed in sheep. Additionally distinct migratory routes for naïve and memory T cells were suggested [6]. Most of our further understanding on T cell migration and proliferation is still based on research in animal models although it is obvious that these models (with a lifespan of several months up to a few years, in pathogen-free conditions) cannot reflect the human exposure to multiple pathogens over decades [26]. Human studies are still limited, as only peripheral blood or tissues from surgical resection material are easily accessible.

The formerly accepted migration model with distinct routes for naïve and memory T cells turns out not to be as segregated as suggested before. T_N cells do not only circulate through blood and lymph between SLOs but also enter non-lymphoid organs, which were first thought to be exclusively confined to memory T cells. T_N cells have recently been identified in non-lymphoid tissue of healthy individuals, representing approximately 10% of the T cells at the intestinal sites (jejunum, ileum and colon) [14]. Additionally, up to 50% of the T cells in non-lymphoid organs of the gut in steady-state mice were shown to express CCR7 (T_N, T_{CM}, and T_{EMRA}) [10]. An immune-surveillance or tolerance-induction role of T_N cells entering the non-lymphoid tissues has been advocated [11]. Although central tolerance during thymic selection eliminates most of the auto-reactive T cells, some of them might escape and migrate to the periphery. In this way, naïve

auto-reactive T cells (RTEs and T_{MN}) could enter healthy tissues; become activated and initiate chronic inflammation. On the other hand, influx of T_N cells in non-lymphoid tissue possibly induces tolerance to organ-specific antigens within the tissue itself [17].

Antigen-presentation to T_N cells takes place primarily in the SLOs. Following antigen recognition, further maturation of T_N cells evolves towards memory T cells, which express tissue-specific surface receptors. T_{CM} cells express the same lymph node homing molecules as T_N cells, namely L-selectin (CD62L) and chemokine receptor 7 (CCR7, also known as CD197), and therefore also have the potential to home to SLOs [27]. T_{EM} cells are $CCR7^-CD62L^-$ and express tissue homing molecules like $\alpha_4\beta_7$ which binds to mucosal adressin-cell adhesion molecule-1 (MAdCAM-1), and CCR9 which interacts with chemokine (C-C motif) ligand 25 (CCL25) in the gut endothelium [12].

T_N cells do usually not express tissue-specific homing receptors on their surface, with exception of moderate levels of $\alpha_4\beta_7$. They enter the lymph node and PPs across specialized post-capillary venules called high endothelial venules (HEV). HEV endothelial cells are cuboidal and are surrounded by multiple layers of fibroblastic reticular cells that produce various extracellular matrix components forming a thick basal lamina. Microscopically they can easily be distinguished from normal venules, which have flat endothelium and a thin basal lamina [28]. HEV endothelium expresses molecules that function in lymphocyte trafficking. This occurs through interaction between surface molecules on the lymphocytes with their HEV-expressing ligand counterparts: CD62L on the T cells with peripheral lymph node addressin (PNAd) and MAdCAM-1; CCR7 on the T cell with CCL21; and lymphocyte function-associated antigen 1 (LFA-1, also known as CD11aCD18) on the T cell with intercellular adhesion molecule 1 (ICAM-1 also known as CD54) (figure 2a) [12,29]. In contrast to HEV in peripheral lymph nodes, HEV in PPs do not express PNAd. In PPs, the tethering/rolling step is mediated by the interaction of CD62L and $\alpha4\beta7$ with MAdCAM, while further adhesion and migration of T-cells happens through LFA-1 and ICAM-1 (figure 2b) [30]. The binding of CD62L to MAdCAM-1 is dependent on the presence of specific carbohydrate ligands on the MAdCAM-1 [31,32]. Evidence supporting this interaction is demonstrated in mice studies, but is still missing for human.

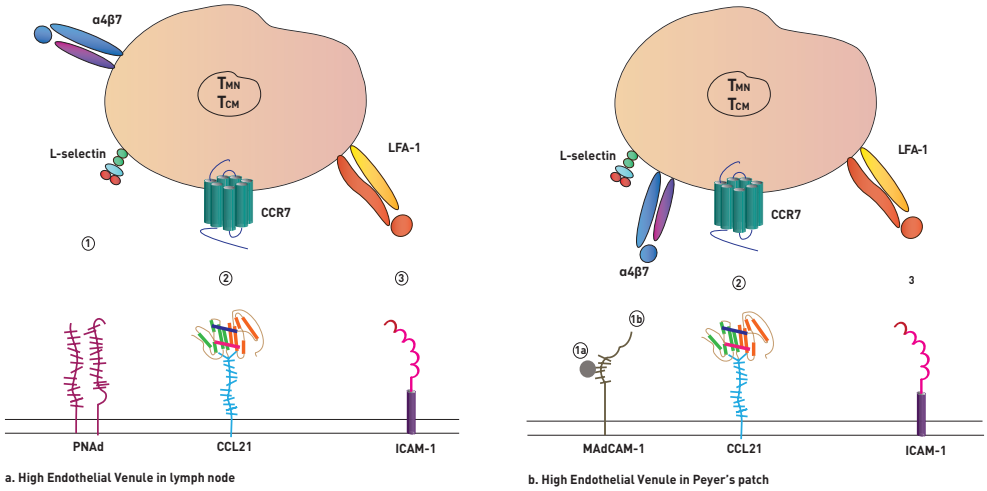

Figure 2. Migration of mature naïve (T_{MN}) and central memory (T_{CM}) T cells during homeostasis.

a. T_{MN} and T_{CM} cells migrate into a lymph node by interaction with surface receptors on high endothelial venules (HEV). (1) CD62L binds with peripheral lymph node addressin (PNAd); (2) chemokine receptor 7 (CCR7) binds with chemokine (c-c motif) ligand 21 (CCL21) and (3) lymphocyte function-associated antigen 1 (LFA-1) binds with intercellular adhesion molecule 1 (ICAM-1).

b. T_{MN} and T_{CM} cells migrate into Peyer's patches by interaction with surface receptors on HEV. Tethering and rolling is mediated by interaction of mucosal addressin-cell adhesion molecule 1 (MAdCAM-1) and CD62L (1a) and αβ7 integrin (1b). Further adhesion and migration occurs through CCR7 with CCL21 (2) and LFA-1 with ICAM-1 (3).

MIGRATION OF NAÏVE T LYMPHOCYTES IN IBD

Tertiary Lymphoid Organs

There is increasing evidence for primary T cell responses independent of SLOs. Chronic inflammation and solid tumours have been found to induce formation of HEV-like vessels in non-lymphoid inflamed tissue and tumour tissue, organizing around so called tertiary lymphoid organs (TLOs) [33-37]. These TLOs are ectopic accumulations of lymphoid cells, arising at a non-lymphoid location, through a process called lymphoid neogenesis. Contrarily, primary and secondary lymphoid organs are found at specific, predefined, sites that seem to be genetically fixed and develop before birth [38]. TLOs morphologically resemble SLOs, since they also have T and B cell compartments, lymphoid chemokines, antigen presenting cells (like dendritic cells) and HEV-like vessels and are therefore also able to recruit T_N cells.

How and why TLO formation occurs is unclear. HEV-like vessels presumably facilitate lymphocyte influx into TLOs, but it is also possible that T_N cells gain initial access to non-

lymphoid tissues via regular blood vessels and that HEVs are formed later in the process [8]. Lymphotoxin $\alpha_1\beta_2$ (LT$\alpha_1\beta_2$, TNF family member) may have a prominent role in regulating the formation of (secondary and tertiary) lymphoid organs and is crucial for HEV development and maintenance [39,40]. After priming, T_N CD4$^+$ cells induce LT$\alpha_1\beta_2$ expression on their surface [41,42]. The presence of ectopic CCL21 on blood vessels during inflammation may trigger the influx of CCR7$^+$ T cells (T_N and T_{CM}). This provides a source of LT$\alpha_1\beta_2$, perpetuating CCL21 expression and facilitating the formation of HEV-like vessels and TLOs. Notably, blocking the lymphotoxin (LT) pathway in colitis mouse models reduced the severity of inflammation and was equally efficacious as anti-TNFα therapy [43,44].

Clinical significance of TLOs has already been described in other diseases like colorectal cancer and rheumatoid arthritis. In early-stage colorectal cancer, TLOs are associated with a better prognosis [45], and are suggested as a possible marker for prognosis [46]. In patients with rheumatoid arthritis, TLOs were present in 49% of synovial tissue, and its presence coincided with longer disease duration and lower response to therapy (more refractory disease) [47]. Reversal of TLOs was a good marker of response to therapy.

Tertiary lymphoid organs in IBD

Recent studies have shown that TLOs might play a role in chronic inflammation of the gut in IBD. Ectopic CCL21 on blood vessels and PNAd-expressing HEV-like vessels has been detected in inflamed gut tissue of patients with ulcerative colitis (UC). A positive correlation between CCL21 expression and influx of T_N cells was observed [8]. CCL21 production was demonstrated in inflamed tissue of patients with Crohn's disease (CD) and UC, and lymphoid aggregates were found in 50% of their intestinal biopsy samples [35]. HEV-like vessels in the active phase of UC and CD were identified only in inflamed gut tissue, compared to a scarce presence of these vessels in non-inflamed colon or during remission of IBD [48,49]. Furthermore, the formation of ectopic lymphoid follicles or TLOs was found in diseased ileal segments of CD patients [50]. In contrast to the chronically inflamed IBD tissue, acute inflammation was not accompanied by the induction of lymphoid aggregates and HEV-like vessels [48]. MAdCAM-1$^+$ intestinal lamina propria venules are increased in UC, both in active disease and remission, compared to normal controls [51]. There was great co-expression of PNAd among MAdCAM-1$^+$ vessels in patients with active disease (66.5%) when compared to patients in remission (7.62%) and PNAd was not detected in the colonic mucosa of normal controls. In a recent study in mice with chronic ileitis, the induction of ectopic lymphoid tissue has been demonstrated in the inflamed intestine, with a statistically significant increase of CCL21 expression and accumulation of T_N cells, T_{EM} and T_{CM} cells in the terminal ileum, compared to healthy wild type mice [15].

Thus far, it remains difficult to distinguish gut-associated lymphoid tissue (GALT) from TLOs and an unambiguous definition is lacking [33]. Nonetheless, chronically inflamed gut induces organized structures that cannot be observed in the gut of healthy controls. These structures,

referred to as TLOs, are present in the deeper layers of the epithelium and contain PNAd-expressing HEV-like vessels, that distinguishes them from the submucosal PPs [48,50,51].

NAÏVE T CELLS IN IBD

Mice: the T cell transfer model

Immunodeficient mice (CB17 severely combined immunodeficient/recombinase activating gene-1$^{-/-}$) develop chronic, severe intestinal inflammation, after transfer of naïve ($CD4^+CD45RB^{high}$) donor T cells into their peritoneal cavity [52]. Co-transferring memory ($CD4^+CD45RB^{low}$) T cells inhibits the development of intestinal inflammation [53] and single transfer of the memory subset does not cause inflammation [54]. One could expect that the transferred T_N cells are primed within GALT and mesenteric lymph nodes (MLNs) before inducing colitis. However, the induction of colitis by transfer of T_N cells was also demonstrated in mice deficient of SLOs (GALT, MLN and spleen) [55]. This study underlines that SLOs are not required for priming T_N cells into colitogenic effector T cells to initiate colitis, leaving the site of priming in these models to be defined. As the pattern of exacerbation and remission of human IBD has not been perfectly phenocopied in these mouse models, the presence and the role of T_N cells in the gut of IBD remains to be elucidated [56].

The potential role of naïve T cells in human IBD

The first suggestions indicating a potential role of (maturation of) T_N cells in the pathogenesis of IBD came from a few (case-)reports showing the effectiveness of thymectomy in adult patients with IBD [57-60]. Thymectomy has been performed in UC patients with thymic hyperplasia and was successful for maintaining remission in patients refractory to conventional therapy [58,60]. Additionally, thymectomy was also performed in a CD patient developing myasthenia gravis, leading to remission of her CD activity [59]. No further publications have arisen on the potential of thymectomy in IBD.

Already in 1995 it was revealed that chronic inflammation in CD induced migration of T_N cells to inflamed sites. The study involved ileal samples of CD patients and bowel samples of controls with colon carcinoma. In the intestines of controls, blood vessels were only found in the lamina propria and beneath the muscularis mucosa, and they exhibited the characteristics of normal (flat) venules. The inflamed intestines of CD patients showed numerous dilated and highly activated small vessels with HEV characteristics, extending from the superficial mucosa layers throughout the deep submucosa. These vessels and the perivascular and mucosal infiltrates all harboured mainly macrophages and T cells. The majority of T cells within the (sub)mucosal infiltrates were T_N ($CD45RA^+CD31^+CD62L^+CD27^+$) and T_{CM} ($CD45RO^+CD31^-CD62L^+CD27^+$) cells. The normal intestinal mucosa of controls was predominantly populated by T_{EM} cells ($CD45RO^+$ $CD31^-CD62L^-$ $CD27^-$) [7].

The expression of maturation markers on $CD4^+$ T cells was analysed in the intestinal mucosa of both CD and UC patients with active disease. Almost all patients were using corticosteroid medication and were included at the time they underwent partial resection of the intestine. Non-involved bowel segments of patients undergoing bowel resection for cancer treatment were used as control. The numbers of $CD45RA^+$ T cells were increased in the intestine of IBD patients (49.0% in UC and 27.7% in CD) as compared to controls (4.5%). The $CD45RA^+$ cells found in IBD patients were mainly located in 'follicular structures' [9].

Another study aimed to characterize T cells present in the intestine of IBD patients before and after therapy. Sigmoid-derived biopsy specimens of IBD patients (CD as well as UC) with distal active colitis were evaluated. Patients that were in remission with mucosal healing showed a reduction of >50% of $CD4^+CD45RA^+$ T cells, compared to inflamed mucosa of patients with active disease. There was no difference in the amount of memory T cells in the inflamed mucosa when compared to non-inflamed mucosa [16].

Regarding RTEs in IBD patients, increased TREC levels in mucosal but not peripheral blood lymphocytes were found in UC patients, indicating direct recruitment of RTEs into the inflamed mucosa [13]. However these results have not yet been duplicated.

We recently studied the maturation of T cells in the gut mucosa in a large group of newly diagnosed, untreated IBD patients. Remarkably high frequencies of T_N were identified in a subgroup of CD as well as UC patients (naïve profile). This group of patients expressed different pro-inflammatory cytokine levels (higher TNF-alpha and lower interferon-gamma) compared to the group of patients with high frequencies of T_{EM}. Furthermore, there was an association between the naïve profile and extended disease location in CD (61). We elaborated on the possible correlation between differences in the maturation of the T cell subsets early in disease course and disease heterogeneity. An extended follow-up of this cohort may elucidate the prognostic value of different maturation profiles at primary diagnosis.

CONCLUSIONS

Contrarily to previous ideas, there is increased evidence that T_N cells migrate to non-lymphoid organs in homeostasis and even more in chronic inflammation. During chronic inflammation, migration of T_N cells could be facilitated by TLOs. Formation of these TLOs appears dependent on $LT\alpha_1\beta_2$; however the signals triggering these mechanisms are waiting to be characterized. Presence of TLOs implies an ongoing recruitment of T_N and T_{CM} cells to the gut mucosa, maintaining a continuous inflammatory process. The relevance of targeted therapy against this phenomenon has not yet been studied. It is conceivable that a process involving dissolution of TLOs could restore immunologic homeostasis.

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3

High endothelial venules associated with T cell subsets in the inflamed gut of newly diagnosed Inflammatory Bowel Disease patients

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SUMMARY

Naive and central memory T lymphocytes (T_N and T_{CM}) can infiltrate the inflamed gut mucosa in Inflammatory Bowel Disease (IBD) patients. Homing of these subsets to the gut might be explained by ectopic formation of tertiary lymphoid organs (TLOs), containing high endothelial venules (HEVs). We aimed to evaluate the presence of HEVs and TLOs in inflamed intestinal mucosa of newly diagnosed, untreated, IBD patients in relation to the presence of T_N and T_{CM} lymphocytes. IBD patients (n=39) and healthy controls (n=8) were prospectively included. Biopsy samples of respectively inflamed and normal intestine were analyzed by immunohistochemistry for lymphocytes (CD3/CD20), blood vessels (CD31) and PNAd expression (MECA-79). T_N and T_{CM} lymphocyte subsets were identified by flowcytometric immunophenotyping. A higher number of HEVs was found in the inflamed colon of patients with ulcerative colitis (median 3.05 HEV/mm^2; (IQR 0-6.39) and ileum of Crohn's disease patients (1.40; 0-4.34) compared to healthy controls (both 0; P=0.033). A high density of colonic HEVs (HEV^{high}) was associated with increased infiltration of T_N and T_{CM} in the inflamed gut (median 87%; IQR 82-93% of T cell population), compared to HEV^{low} patients (58%; 38-81%; P=0.003). The number of colonic follicles was higher in HEV^{high} patients (median 0.54/mm^2; IQR 0.28-0.84) compared to HEV^{low} patients (0.25/mm^2; 0.08-0.45; P=0.031) and controls (0.31/mm^2; 0.23-0.45; P=0.043). Increased homing of T_N and T_{CM} lymphocytes to inflamed gut tissue in IBD patients might be facilitated by ectopic formation of extrafollicular HEVs and TLOs in a subgroup of patients.

INTRODUCTION

Lymphocyte activation and homing of lymphocytes to target organs are key phenomena in chronic inflammation, including Inflammatory Bowel Diseases (IBD). Lymphocyte activation is triggered by interaction of antigen receptors with cognate antigens. The initial priming of naive T lymphocytes (T_N) occurs in secondary lymphoid organs (SLOs; spleen, lymph nodes, gut associated lymphoid tissue (GALT; Peyer's patches, isolated lymphoid follicles and appendix)). SLOs are genetically encoded, specialized environments for optimal antigen presentation, and lymphocyte activation. Upon antigen priming in the SLO, activated T lymphocytes migrate to non-lymphoid tissue sites, where they may exercise effector (proinflammatory or regulatory) functions upon renewed antigen encounter. It is well known that activated effector memory T lymphocytes (T_{EM}) travel to the gut mucosa following $\alpha4\beta7$/MAdCAM-1 and CCR9/CCL25 interaction at the level of post-capillary venules [1,2].

In physiological conditions, T_N and central memory T lymphocytes (T_{CM}) are thought to migrate exclusively to SLOs [1,3]. Specialized vessels, so-called high endothelial venules (HEVs), direct tissue migration of circulating T_N and T_{CM} into SLOs. This process is guided by different vascular addressins, such as MAdCAM-1 and peripheral lymph node addressin (PNAd). PNAd on endothelial cells is the ligand of L-selectin, which is expressed on T_N and T_{CM} lymphocytes, while practically absent on the majority of T_{EM} or effector memory T cells re-expressing CD45RA (T_{EMRA}). PNAd can be identified by the monoclonal antibody MECA-79 [4,5]. Distribution of these addressins differs between peripheral lymph nodes, mesenteric lymph nodes and Peyer's patches. MAdCAM-1 is constitutively displayed on HEVs in Peyer's patches, on mesenteric lymph nodes and on flattened venular endothelial cells in the intestinal lamina propria [6]. PNAd is predominantly present on HEVs in peripheral lymph nodes, mesenteric lymph nodes and at very low levels on the abluminal side of HEVs in Peyer's patches [4].

We and others have demonstrated the presence of T_N and T_{CM} lymphocytes in non-lymphoid gut tissue [7-11]. In our previous work we identified three subgroups of newly diagnosed IBD patients with either increased percentage of mucosal T_N, T_{CM} or T_{EM} [7]. It is still unclear how homing of these T_N and T_{CM} lymphocytes to non-lymphoid tissue is established.

Processes occurring during chronic inflammation, and formation of solid tumours, were shown to induce ectopic formation of HEV-like vessels, in inflamed non-lymphoid and tumour tissue, constituting the so-called tertiary lymphoid organs (TLOs) [12-15]. Little is known about $PNAd^+$ HEV-like vessels in IBD pathogenesis (reviewed in [16]), since published data is limited to patients with long-standing disease under anti-inflammatory treatment [5,17].

We aimed to investigate the presence of HEVs and their relation to lymphoid follicles in the inflamed intestinal mucosa of newly diagnosed, untreated, IBD patients. Furthermore, since T_N and T_{CM} lymphocytes are known to express L-selectin [7,18,19], we aimed to determine whether the infiltration of these cells is correlated with the presence of HEVs and TLOs in the inflamed intestinal mucosa.

MATERIAL AND METHODS

Patients and samples

At the IBD centre of the Rijnstate Hospital, Arnhem, The Netherlands, 39 newly diagnosed adult IBD patients and eight healthy controls were prospectively included in the study. The diagnosis of IBD was based on a combination of clinical, endoscopic, histologic and radiologic internationally accepted criteria. Intestinal biopsy specimens of the macroscopically most inflamed ileal and colonic mucosa (when ulcerations were present these biopsy specimens were taken from the edge of this ulceration) were obtained at the moment of diagnosis, before the initiation of any medical treatment. Thus, when disease was located in the ileum, only ileal biopsies were analysed; when disease was located in the colon, only colonic biopsies were analysed and when the disease was located in ileum and colon both ileal and colonic biopsies were analysed. Besides regular histopathological analysis, four samples were used to immunophenotype the mucosal lymphocyte population. Patient characteristics were documented and disease phenotype was assessed.

Ileal and colonic biopsy specimens were obtained from eight control subjects undergoing ileocolonoscopy for iron deficiency anaemia or polyp surveillance. Biopsy specimens of controls were taken from macroscopically non-affected, non-inflamed ileal and colonic areas. Regular histopathological examination confirmed that these areas had no signs of inflammation. Besides regular histopathological analysis, four samples were used to immunophenotype the mucosal lymphocyte population. Parallel venous blood samples were drawn following endoscopic evaluation at primary diagnosis. Serum samples were coded and stored at -20°C until analysis.

Immunohistochemistry

Immunohistochemistry was carried out on 3 µm thick sections from formalin-fixed, paraffin-embedded archived blocks of biopsied specimen of ileal and colonic mucosa. The following monoclonal antibodies were used for immunostaining: MECA-79 (identifies 6-sulfo sialyl Lewis on core-1 branched O-linked sugars (PNAd) which binds L-selectin, Sanbio, Uden, The Netherlands), CD31 (monoclonal, identifies human CD31 to demonstrate endothelial cells, Sanbio, Uden, The Netherlands), CD3 (clone LN10, identifies human CD3 to demonstrate T-cells, Leica Microsystems B.V., Eindhoven, The Netherlands) and CD20 (clone L26, identifies human CD20 to demonstrate B-cells, Leica Microsystems B.V., Eindhoven, The Netherlands). Slides were incubated with the primary antibody in an automatic immunostainer (Bond, Leica biosystems Nussloch GmbH 2016, Eindhoven, The Netherlands).

Stained slides were scanned with an Intellisite high-resolution scanner (Philips ultra-fast scanner 1.6 RA, Philips Digital Pathology, Best, The Netherlands) and analysed with the Intellisite IMS image analyser. Quantification was done by two independent observers who were blinded to the clinical outcome and flowcytometric analyses. The total surface area of

each slide was measured in square millimetre (mm^2). Absolute numbers of $CD31^+$ vessels and MECA-79^+ HEVs were determined by optical counting of vessel numbers on all slides. The location of HEVs relative to lymphoid follicles was determined on CD3 and CD20 immunostained sections and absolute numbers of intra- and extrafollicular HEVs were quantified. The density of extrafollicular HEVs (MECA-79^+ vessels/mm^2 excluding the surface of follicles) and the ratio of HEVs among all vessels (MECA-79^+ vessels/$CD31^+$ vessels) were calculated. In malignancy, a cut-off point for increased density of HEVs has been described in patients with breast cancer [20]. However, in chronic inflammation, no such approach has been undertaken. We discriminated patients with a high and a low density of HEVs using a cut-off point set at the median density of extrafollicular HEVs/mm^2 in the inflamed colon of UC patients.

Presence of inflammation was established for all sections based on known architectural changes and inflammatory features such as crypt distortion, decreased crypt density, erosions, ulcers, increased lamina propria cellularity, crypt abscesses and epithelioid granuloma. Lymphoid follicles were defined as a dense nodular lymphocyte aggregates containing B and T cell compartments. Their presence was assessed on CD3 and CD20 immunostained sections. The number of follicles was counted. In absence of a clear-cut definition of TLOs in the gut, we defined TLO as additional follicles in patients compared with the number of follicles in controls. Furthermore, it is our interpretation that the presence of extrafollicular HEVs represent early phase of lymphoid neogenesis (TLOs).

Immunophenotyping the mucosal lymphocytes

Intestinal biopsy specimen were sampled and analysed as described earlier [7]. Briefly, besides the standard histopathological analysis of biopsy specimens, four samples of the most inflamed regions of colon and ileum were used for flowcytometric immunophenotyping. Biopsy specimen were kept in phosphate-buffered saline solution at 2-8°C and processed within 8 hours. Specimens were finely minced in Hanks'/1% bovine serum albumin using a 70-mm gaze and spatula followed by Ficoll density gradient centrifugation.

The homogenate was resuspended, after washing, in 0.5 mL Hanks'/1% bovine serum albumin. The cell number was estimated by microscopic counting with a KOVA glasstic slide (Hycor Biomedical Ltd., Penicuik, United Kingdom). For flow cytometric analysis 200 mL of the total cell suspension was used, disregarding absolute cell number. Single cell suspensions of the biopsies were analysed using a FACS Canto (BD Biosciences). Mucosal lymphocyte subpopulations were differentiated: B cells ($CD19^+$), T cells ($CD3^+$), regulatory T cells ($CD3^+CD25^{high}FoxP3^+$), mucosal T cells ($CD3^+CD103^+$), T-helper cells ($CD3^+CD4^+$) and cytotoxic T cells ($CD3^+CD8^+$) and expressed as percentages of the whole lymphocyte population (CD45+/low side scatter). The maturation state of T cells was assessed by using CD45RA and CD27 [21], defining naive T cells (T_N: $CD45RA^+CD27^+$), central memory T cells (T_{CM}: $CD45RA^-CD27^+$), effector memory T cells (T_{EM}: $CD45RA^-CD27^-$) and T effector memory cells re-expressing CD45RA (T_{EMRA}: $CD45RA^+CD27^-$). We also analysed groups of T cells according to their known

migration phenotypes. T_N and T_{CM} lymphocytes are known to enter HEVs through the ligand PNAd, as they are L-selectin$^+$ [18,19] and were analysed both independently as well as taken together. The same analysis was performed for T_{EM} and T_{EMRA} lymphocytes, which are known to be L-selectin$^-$ [18,19]

Multiplex immunoassay

In a subgroup of the current patient cohort we analysed serum chemokines using Multiplex immunoassays, including CXCL-13 and CCL-19, which are known to be required for lymphoid organogenesis [22]. These assays were performed at the MultiPlex Core Facility of the Laboratory of Translational Immunology (UMC Utrecht, The Netherlands) using an in-house validated platform (ISO9001). Briefly, color-coded magnetic beads (MagPlex Microsperes, Luminex, Austin, Texas) were conjugated to analyte-specific antibodies and incubated with standard dilutions or sample for 1 hour during continuous shaking in the dark. Pre-treatment of samples with HeteroBlock (Omega biologicals, Inc., Bozeman, Montana, USA) was performed, to prevent interference by binding of heterophilic antibodies. Plates were washed (Bio-Plex Pro II Wash Station; Bio-Rad, Hercules, California, USA) and a corresponding cocktail of biotinylated detection antibodies was added followed by streptavidin-phycoerythrin (PE) incubation. Fluorescence intensity of PE was measured with a Flexmap 3D system (Luminex) and analysed by BioPlex Manager Software (version 6.1; Bio-Rad, USA) using 5-parameter curve fitting.

Statistical analysis

Data of ileal biopsy specimens of CD patients was compared to ileal biopsy specimens of healthy controls. The data of colonic biopsy specimens of UC and CD patients were compared to the colonic biopsy specimens of healthy controls. Ileum and colon were not mutually compared because of anatomical and physiological differences, including the influence of the microbiome. Data was collected and analysed with SPSS statistics (version 21.0.0.0; IBM Corp). Differences between groups were tested using non-parametric tests. Continuous variables were described as median with interquartile range (IQR, 1st quartile-3rd quartile). Mann-Whitney *U* test was used to identify differences in continuous variables. Categorical variables were described as absolute frequencies. Kruskal-Wallis analysis was used followed by the Dunn test when applicable. Spearman rank correlation was performed to measure the degree of association between variables. Statistical significance was defined as a *P*-value less than 0.05.

Ethical Considerations

Written informed consent was obtained from each participating patient. The procedures were performed in accordance with the Declaration of Helsinki. The regional medical ethics committee approved the protocol, according to Dutch Law (NL28761.091.09).

RESULTS

Ten newly diagnosed patients with ulcerative colitis (UC) and 29 newly diagnosed IBD patients with Crohn's disease (CD) were included in this study. In addition eight healthy controls were studied. Baseline characteristics of patients are outlined in table 1.

Table 1. Baseline characteristics.

	CD (n=29)	CU (n=10)
Age at diagnosis in years, median (IQR)	28 (20-44)	28 (20-35)
Female, n (%)	21 (72%)	7 (70%)
Symptoms before diagnosis in months		
• 0-3	10 (35%)	7 (70%)
• 3-6	9 (31%)	1 (10%)
• >6	10 (35%)	2 (20%)
Follow-up period in months, median (IQR)	55 (23-62)	44 (22-48)
Family with IBD, n (%)	4 (14%)	4 (40%)
Smoking status		
• Never	17 (59%)	8 (89%)
• Current	10 (34%)	1 (11%)
• Ceased	2 (7%)	-
• Unknown	-	-
HBI score, median (IQR)	9 (6-13)	-
CD disease location (Montreal)		-
• Ileal, n (%)	8 (28%)	
• Colonic, n (%)	5 (17%)	
• Ileocolonic, n (%)	16 (55%)*	
• + Upper GI involved, n (%)	10 (39%)	
• + Perianal disease, n (%)	5 (17%)	
CD disease behaviour (Montreal)		-
• Inflammatory, n (%)	21 (72%)	
• Stricturing, n (%)	7 (24%)	
• Penetrating, n (%)	1 (4%)	
SES-CD score, median (IQR)	14 (8-22)	-
SES-CD severity		-
• Mild (4-10), n (%)	11 (38%)	
• Moderate (11-19), n (%)	9 (31%)	
• Severe (>19), n (%)	9 (31%)	

Table 1. continued

	CD (n=29)	CU (n=10)
UC clinical score (Montreal)	-	
• Remission, n (%)		-
• Mild, n (%)		1 (10%)
• Moderate, n (%)		4 (40%)
• Severe, n (%)		5 (50%)
UC disease location (Montreal)	-	-
• Proctitis, n (%)		-
• Left-sided, n (%)		-
• Pancolitis, n (%)		10 (100%)
UC endoscopic severity (Mayo)	-	
• Normal, n (%)		-
• Mild, n (%)		2 (20%)
• Moderate, n (%)		4 (40%)
• Severe, n (%)		4 (40%)

CD = Crohn's disease, UC = ulcerative colitis, IQR = interquartile range, IBD = inflammatory bowel disease, HBI= Harvey-Bradshaw Index, SES-CD = simple endoscopic score for Crohn's disease

* One patient was excluded from the analyses of colon biopsies because of technical failure of MECA-79 staining.

Number of blood vessels and follicles was comparable in patients and controls

In each sample, we determined the absolute number of blood vessels ($CD31^+$ vessels and $MECA\text{-}79^+$ vessels, hereafter denoted as HEVs) and follicles by immunohistochemical staining. HEVs were determined by optical counting of $MECA\text{-}79^+$ vessel numbers on all slides. The location of these HEVs relative to lymphoid follicles was determined on CD3 and CD20 immunostained sections and absolute numbers of intra- and extrafollicular HEVs were quantified. The density of extrafollicular HEVs ($MECA\text{-}79^+$ vessels/mm^2 excluding the surface of follicles) and the ratio of HEVs among all vessels ($MECA\text{-}79^+$ vessels/$CD31^+$ vessels) were calculated. Overall, we observed high heterogeneity in patient results. No statistical significant difference was found when comparing the entire patient group and controls for the number of vessels or follicles in biopsy specimen (see table 2). In addition, the number of HEVs within these follicles was comparable. However, in UC there was a trend towards more HEVs in the inflamed colon (4.80/mm^2, (0.45-6.14)) than in the colon of healthy controls (0.71/mm^2, (0-1.85), P=0.109).

Table 2. Blood vessels and follicles in gut mucosa of IBD patients and controls.

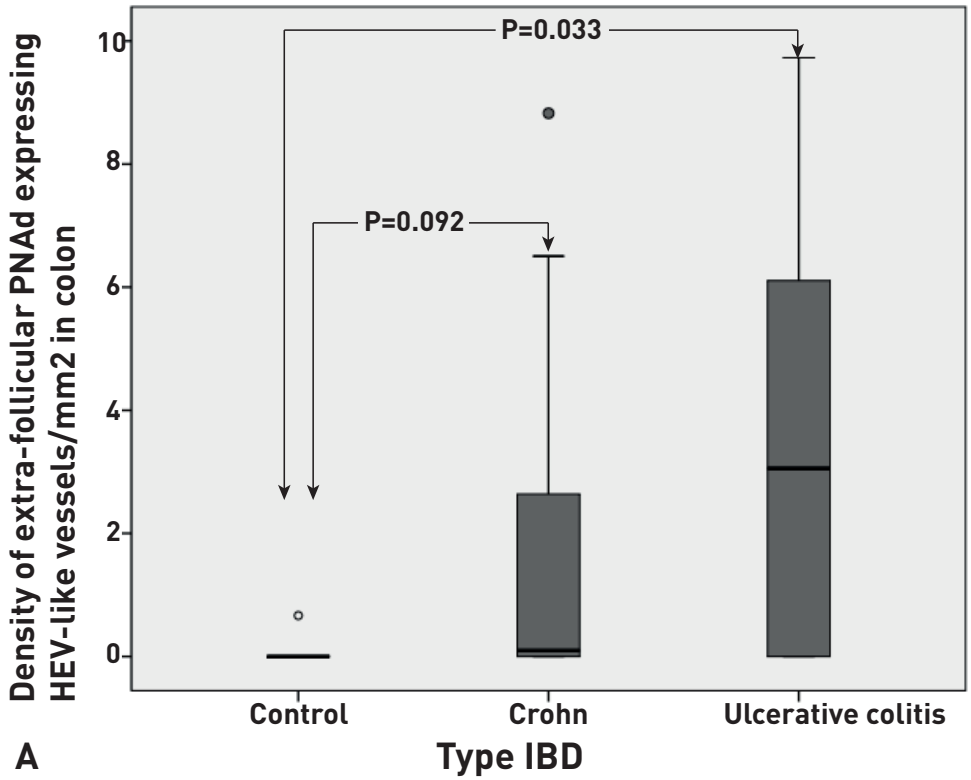
	CD (inflamed)	UC (inflamed)	Healthy controls (non-inflamed)	*P*-value
Number of CD31$^+$ vessels/mm^2 colon, median (IQR)	84 (60-107)	125 (63-139)	71 (43-89)	NS
Number of CD31$^+$ vessels/mm^2 ileum, median (IQR)	93 (70-114)	-	87 (69-102)	NS
Number of HEVs/mm^2 colon, median (IQR)	0.35 (0-5.17)	4.80 (0.45-6.14)	0.71 (0-1.85)	NS
Number of HEVs/mm^2 ileum, median (IQR)	1.58 (0.34-6.69)	-	1.23 (0-5.19)	NS
% MECA-79 expressing CD31$^+$ vessels colon, median (IQR)	0.85 (0-4.80)	4.63 (0.38-12.24)	0.79 (0-2.59)	NS
% MECA-79 expressing CD31$^+$ vessels ileum, median (IQR)	2.02 (0.39-6.82)	-	1.02 (0-8.99)	NS
Colonic follicles/mm^2 tissue, median (IQR)	0.27 (0.12-0.51)	0.51 (0-0.82)	0.31 (0.23-0.45)	NS
Ileal follicles/mm^2 tissue, median (IQR)	0.35 (0.15-0.57)	-	0.37 (0-0.55)	NS

CD = Crohn's disease, UC = ulcerative colitis, IQR = interquartile range

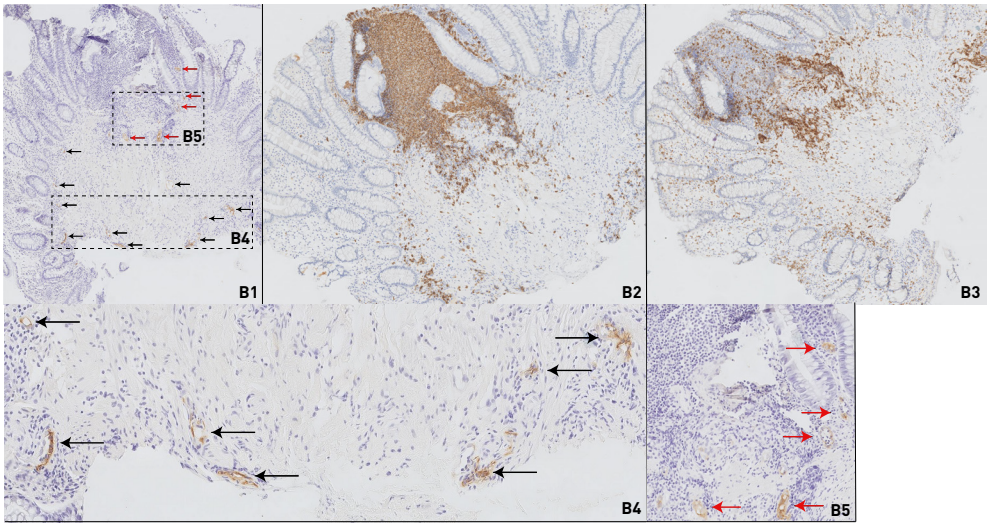
Increased extrafollicular HEV expression in IBD patients compared to controls

Next, we calculated the percentage and density of extrafollicular HEVs and found that colon samples of healthy controls did not show any, whereas the inflamed colon of UC patients showed a high percentage (median 3.71% (IQR 0-6.23%), *P*=0.033, figure 1) and a high median density (3.05 /mm^2 (IQR 0-6.39), *P*=0.033) of extrafollicular HEVs. In the inflamed colon of CD patients, we observed a trend towards a higher number of extrafollicular HEVs (median 0.10 (IQR 0-2.98),) compared to healthy controls (median 0 (IQR 0-0), *P*=0.092).

In the ileum of CD patients, the percentage of extrafollicular HEVs was higher in inflamed ileum (median 1.17% (IQR 0-3.77%)) compared to ileum of healthy controls (median 0% (IQR 0-0.4%)) (*P*=0.028). These samples showed a median density of 1.40 extrafollicular HEVs/mm^2 (IQR 0-4.34), whereas in healthy controls only a few HEVs/mm^2 (0%, IQR 0-0.50) were observed (*P*=0.033). The presence of extrafollicular HEVs in the inflamed ileum and colon of IBD patients might represent an early phase of TLO formation.


Density of extra-follicular PNAd expressing HEV-like vessels/mm2 in colon
10
8
6
4
2
0
P=0.033
P=0.092
Control
Crohn
Ulcerative colitis
Type IBD

A


B5
B4
B1
B2
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B

Figure 1. HEVs in inflamed colonic tissue of patients with inflammatory bowel disease (IBD).

Density of extrafollicular HEVs in colonic tissue (A), determined in the inflamed colonic tissue of patients with Crohn's disease (CD) and ulcerative colitis (UC) as well as in healthy controls. The density is higher in UC patients when compared to controls (*P* =0.033) and there is a trend for higher densities in CD patients compared to controls (*P* =0.092).
Immunohistochemical staining (B1-B5). Representative photomicrographs, with a magnification of 200x, of an immunostained colonic biopsy sample from a Crohn's disease patient with MECA-79 staining (B1), CD20 staining showing B lymphocytes (B2) and CD3 staining showing T lymphocytes (B3). HEVs are shown in closer detail in B4 (extrafollicular) and B5 (inside a follicle). Arrows in figure B1, B4 and B5 indicate HEVs. Red arrows indicate HEVs inside a lymphoid follicle and black arrows indicate extrafollicular HEVs.

HEV density is not associated with neovascularization

To examine whether the difference in density of PNAd-expressing HEV-like vessels in patients could be attributed to neovascularization, we quantified the number of HEVs/mm^2 and correlated this with the total number of CD31$^+$ vessels in patients and controls. We also compared colonic UC with colonic CD biopsy specimens. We did not find a correlation and no statistical difference was found between UC and CD patients (*P*=0.198), indicating that the differences in HEV density was not related to or associated with neovascularization.
To assess a potential correlation with several confounders, as listed in table 1, we found that HEV density in inflamed colon was negatively correlated with smoking (Spearman's rho= -0.543, *P*=0.002) when analysing all IBD patients. However, when subdivided into CD and UC, it was only significantly correlated in CD (Spearman's rho -0.683, *P*=0.001) and not in UC (Spearman's rho -0.139, *P*=0.721). Smoking was not correlated with HEV density of inflamed ileum in CD patients. We did not find any correlations with other baseline characteristics (data not shown).

HEV^{high} IBD patients have increased colonic lymphoid follicles and infiltration of T_N+T_{CM} cells

The immunohistochemical findings were correlated to the maturation state of T cell infiltrates, assessed by flowcytometric immunophenotyping of biopsied specimens taken from the same area of inflamed tissue and harvested during the same ileocolonoscopy. The extrafollicular HEV density in inflamed colon of IBD patients (n=30, 10 UC patients, 20 CD patients) was positively correlated with the percentage of mucosal T_N+T_{CM} lymphocytes (Spearman's rho= +0.544, *P*=0.002) and negatively correlated with the percentage of mucosal T_{EM}+T_{EMRA} lymphocytes (Spearman's rho= -0.545, *P*=0.002). Comparable percentages of lymphocyte subsets were found for UC and CD patients (T_N+T_{CM} lymphocytes (*P*=0.406), T_{EM}+T_{EMRA} lymphocytes (*P*=0.451)).
In malignancy, a cut-off point for increased density of HEVs has been described in patients with breast cancer [20]. However, in chronic inflammation, no such approach has yet been undertaken. We discriminated patients with a high and a low density of HEVs using a cut-off point set at the median density of extrafollicular HEVs/mm^2 in the inflamed colon of UC patients. According to this cut off value, we identified 10 HEV^{high} patients and 20 HEV^{low} patients (table 3). HEV^{high} patients displayed less cytotoxic T cells (*P*=0.00008), a higher CD4:CD8 ratio (*P*=0.0008), less

mucosal T cells (P=0.015), more T_{CM} cells (P=0.0009), more T_N+T_{CM} cells (P=0.003), less T_{EM} cells (P=0.022), less T_{EMRA} cells (P=0.008) and less T_{EM}+T_{EMRA} cells (P=0.004) in the inflamed colonic mucosa than HEV^{low} patients (table 3 and figure 2).

The number of colonic follicles was higher in HEV^{high} IBD patients (median 0.54/mm^2, (IQR 0.28-0.84)) compared to HEV^{low} patients (median 0.25/mm^2, (IQR 0.08-0.45) P=0.031) and to healthy controls (0.31/mm^2, (IQR 0.23-0.45), P=0.043). The number of colonic follicles in the inflamed colon of patients was comparable between CD and UC (P=0.348).

In a subgroup of the current patient cohort we analysed a series of serum cytokines and chemokines of which CXCL-13 and CCL-19 (both associated with lymphoid organogenesis [22]) were present at higher concentrations in the HEV^{high} patient group. Patients in this group (n=4) had higher baseline levels of CXCL-13 (median 67 pg/ml (IQR 52-742 pg/ml)) compared to patients in the HEV^{low} group (n=8, median 36 pg/ml (IQR 27-47 pg/ml) P=0.008) and to healthy controls (n=4, median 24 pg/ml (IQR 18-28 pg/ml) P=0.029, figure 3). Furthermore, there was a trend towards higher CCL-19 levels in HEV^{high} patients (median 176 pg/ml, IQR 126-199 pg/ml) compared to HEV^{low} patients (median 125 pg/ml, IQR 90-141 pg/ml, P=0.073).

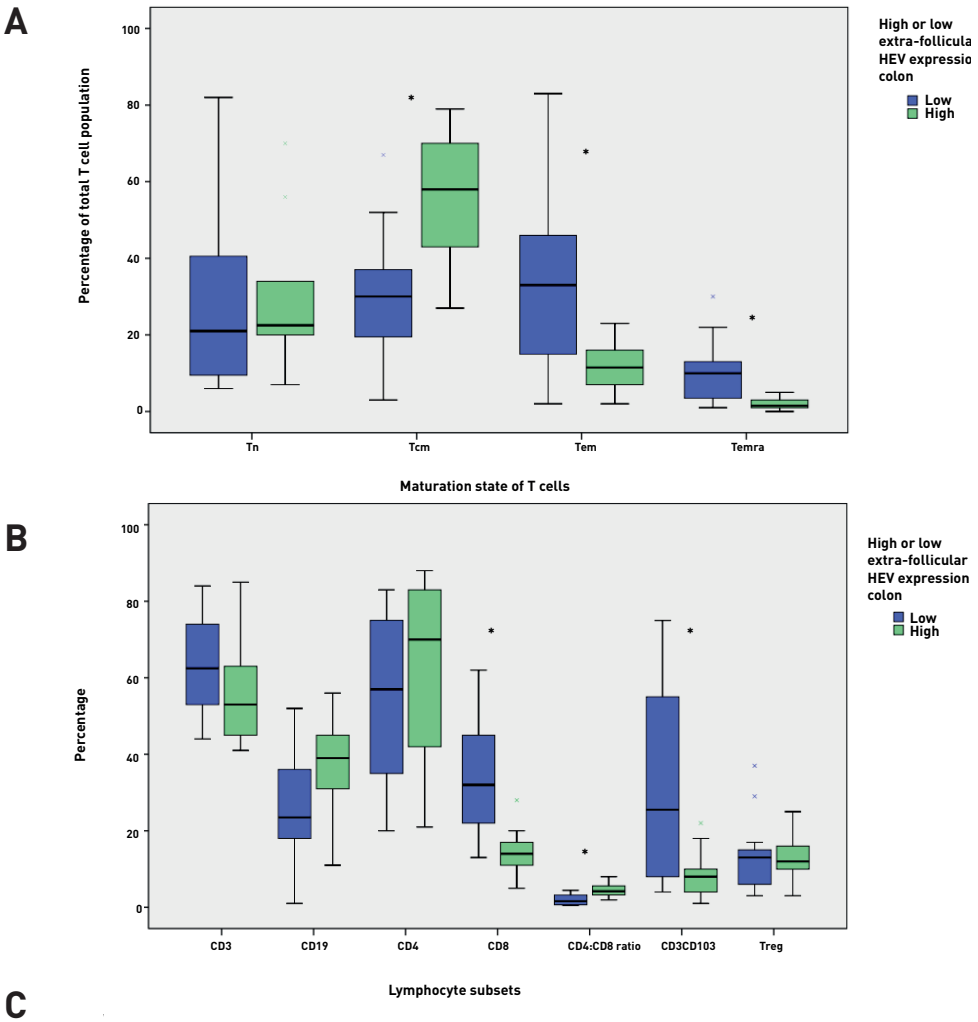
Table 3. Characteristics and FACS results of HEV^{high} and HEV^{low} IBD patients.

	HEV^{high} (n=10)	**HEV^{low} (n=20)**	***P*-value**
CD/UC	5/5	15/5	0.169
Age at diagnosis in years, median (IQR)	21 (20-36)	28 (19-33)	NS
Female, n (%)	9 (90%)	14 (70%)	NS
Symptoms before diagnosis in months			NS
0-3	4 (40%)	12 (60%)	
3-6	5 (50%)	3 (15%)	
>6	1 (10%)	5 (25%)	
Follow-up period in months, median (IQR)	34 (22-56)	50 (28-65)	NS
Family with IBD, n (%)	3 (30%)	5 (25%)	NS
Smoking status			
• Never	9 (90%)	11 (55%)	0.029
• Current	-	8 (40%)	
• Ceased	-	1 (5%)	
• Unknown	1 (10%)	-	
HBI score, median (IQR)	7 (5-14) (n=5)	10 (7-13) (n=15)	NS
CD disease location (Montreal)			NS
• Ileal, n (%)	-	-	
• Colonic, n (%)	2 (40%)	3 (20%	
• Ileocolonic, n (%)	3 (60%)	12 (80%)	
• + Upper GI involved, n (%)	1 (10%)	7 (44%)	
• + Perianal disease, n (%)	2 (20%)	3 (17%)	

Table 3. continued

	HEV^{high} (n=10)	HEV^{low} (n=20)	*P*-value
CD disease behaviour (Montreal)			NS
• Inflammatory, n (%)	5 (100%)	11 (73%)	
• Stricturing, n (%)	-	3 (20%)	
• Penetrating, n (%)	-	1 (7%)	
SES-CD score, median (IQR)	14 (9-19) (n=5)	20 (12-34) (n=15)	NS
SES-CD severity			NS
• Mild (4-10), n (%)	2 (40%)	2 (13%)	
• Moderate (11-19), n (%)	3 (60%)	5 (33%)	
• Severe (>19), n (%)	0 (0%)	8 (53%	
UC clinical score (Montreal)			NS
• Remission, n (%)	-	-	
• Mild, n (%)	1 (20%)	-	
• Moderate, n (%)	2 (40%)	2 (40%)	
• Severe, n (%)	2 (40%)	3 (60%)	
UC disease location (Montreal)			NS
• Proctitis, n (%)	-	-	
• Left-sided, n (%)	-	-	
• Pancolitis, n (%)	5 (100%)	5 (100%)	
UC endoscopic severity (Mayo)			NS
• Normal, n (%)	-	-	
• Mild, n (%)	2 (40%)	-	
• Moderate, n (%)	1 (20%)	3 (60%)	
• Severe, n (%)	2 (40%)	2 (40%)	
FACS analysis of inflamed colon*			
• CD3, median (IQR)	50 (45-63)	59 (52-72)	0.267
• CD19, median (IQR)	39 (29-49)	23 (18-42)	0.108
• CD4, median (IQR)	74 (38-83)	61 (36-75)	0.169
• CD8, median (IQR)	15 (11-19)	29 (22-37)	0.00008
• CD4/CD8 ratio, median (IQR)	4.3 (3.0-6.1)	2.2 (1.2-3.2)	0.0008
• CD103, median (IQR)	7 (4-12)	15 (8-43)	0.015
• T_N, median (IQR)	23 (20-40)	23 (9-54)	0.779
• T_{CM}, median (IQR)	58 (41-72)	33 (20-45)	0.001
• T_{EM}, median (IQR)	12 (6-18)	26 (12-46)	0.024
• T_{EMRA}, median (IQR)	2 (1-3)	6 (2-12)	0.010
• Treg, median (IQR)	12 (8-18)	14 (12-17)	0.379

CD = Crohn's disease, UC = ulcerative colitis, HEV = high endothelial venule, IQR = interquartile range, IBD = inflammatory bowel disease, HBI= Harvey-Bradshaw Index, SES-CD = simple endoscopic score for Crohn's disease, T_N = naïve T lymphocyte, T_{CM} = central memory T lymphocyte, T_{EM} = effector memory T lymphocyte, T_{EMRA} = effector memory T lymphocyte re-expressing RA, Treg= regulatory T lymphocyte
*Expressed as percentage of the whole lymphocyte population


A
Percentage of total T cell population
100
80
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20
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Tn
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Maturation state of T cells
High or low extra-follicula HEV expressio colon
Low
High
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Percentage
100
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CD3
CD19
CD4
CD8
CD4:CD8 ratio
CD3CD103
Treg
Lymphocyte subsets
High or low extra-follicular HEV expression colon
Low
High
C
Temra 2%
Tnaive 54%
Tem 6%
Tcm 38%
CD45RA PE-A
CD27 PerCP-Cy5-5-A
C1
Temra 5%
Tnaive 3 %
Tem 91%
Tcm 2%
CD45RA APC-A
CD27 PerCP-A
C2

Figure 2. Correlation between high endothelial venules (HEVs) and T cell subpopulations.

Lymphocyte subsets as well as maturation state of T lymphocytes was assessed with flowcytometric immunophenotyping of biopsied specimens taken from the same inflamed colonic tissues during the same ileocolonoscopy. Patients are divided in HEV^{high} (n=10) or HEV^{low} (n=20) group using the median density of extrafollicular PNAd expressing HEV like vessels in inflamed colon of ulcerative colitis (UC) patients as cut off value. Statistical significant results are indicated with an asterisk (*).

A. Shows more central memory T cells (T_{CM}, $CD45RA^{-}CD27^{+}$, p=0.0009), less effector memory T cells (T_{EM}, $CD45RA^{-}CD27^{-}$, p=0.022) and less effector memory T cells re-expressing RA (T_{EMRA}, $CD45RA^{+}CD27^{-}$, p=0.008) in HEV^{high} patients when compared with HEV^{low} patients. There was no statistical significant difference for naive T cells (T_{N} $CD45RA^{+}CD27^{+}$).

B. Shows less cytotoxic T cells ($CD3^{+}CD8^{+}$, p=0.00008), a higher CD4:CD8 ratio (p=0.0008) and less mucosal T cells ($CD3^{+}CD103^{+}$, p=0.015) in HEV^{high} patients. There was no statistical significant difference for B cells ($CD19^{+}$), T cells ($CD3^{+}$), T-helper cells ($CD3^{+}CD4^{+}$) and regulatory T cells ($CD3^{+}CD25^{high}FoxP3^{+}$).

C. Representative flow cytometric dot plots from inflamed colonic biopsy specimens of IBD patients which show the distribution of the maturation of ($CD3^{+}$) T lymphocytes. C1 shows a HEV^{high} patient with mainly T_{N} ($CD45RA^{+}CD27^{+}$, 54%) and T_{CM} ($CD45RA^{-}CD27^{+}$, 38%) lymphocytes. C2 shows a HEV^{low} patient with mainly (91%) T_{EM} ($CD45RA^{-}CD27^{-}$) lymphocytes.

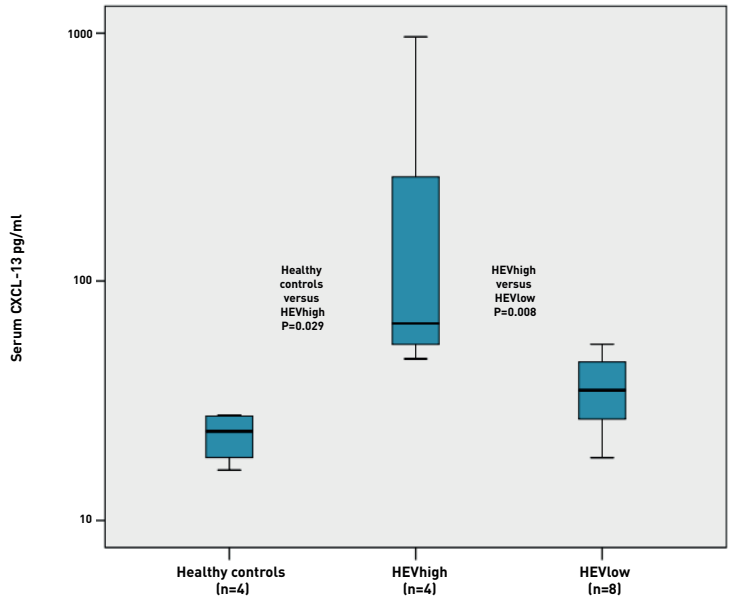

Figure 3 Serum CXCL-13 concentrations in IBD patients and healthy controls.

Serum of a subgroup of patients and healthy controls was analysed by multiplex immunoassay for several cytokines and chemokines. Patients are divided in HEV^{high} or HEV^{low} group using the median density of extrafollicular HEVs in inflamed colon of ulcerative colitis patients as cut off value. HEV^{high} patients had higher CXCL-13 concentrations compared to HEV^{low} patients (P=0.008) and compared to healthy controls (P=0.029).

DISCUSSION

In the present study, increased percentages of extrafollicular HEVs were observed in inflamed colon and ileum of newly diagnosed IBD patients compared to healthy controls. In controls, these HEVs were restricted to lymphoid follicles (SLOs). A high heterogeneity was observed in the number of extrafollicular HEVs in IBD patients. We identified two subgroups of patients according to the density of the extrafollicular HEVs in the inflamed colonic mucosa: HEV^{high} and HEV^{low}. In HEV^{high} patients, a higher percentage of T_N+T_{CM} lymphocytes was observed in the inflamed colonic mucosa compared to HEV^{low} patients. Patients in the HEV^{high} group also displayed a higher number of lymphoid follicles (TLOs) in the inflamed colonic mucosa compared to patients in the HEV^{low} group and controls. Furthermore, chemokines associated with TLO formation (CXCL-13 and CCL-19) were upregulated in the serum of HEV^{high} patients
In physiologic conditions, HEVs are known to be exclusively present within SLOs, whereas in the present study we demonstrated their presence outside follicles in the inflamed intestine of IBD patients. It is our interpretation that extrafollicular HEVs represent an early phase of lymphoid neogenesis (TLO formation), as these vessels were virtually absent in healthy controls. In addition to Peyer's patches, GALT also contains other forms of SLOs, such as isolated lymphoid follicles and submucosal lymphocyte aggregations. Isolated lymphoid follicles are mostly seen within the mucosa, while submucosal lymphocyte aggregations lie deeper within the muscularis mucosa and submucosa [23,24]. Peyer's patches are mostly present in jejunum and ileum while isolated lymphoid follicles have also been found in the human colon [24]. In accordance with this, we found HEVs within lymphoid follicles of the normal gut mucosa of both ileum and colon of healthy controls; however, they did not display such vessels outside these follicles.
In IBD, HEVs were previously investigated in one study using MECA-79 immunostaining of biopsy specimens from UC patients and surgical specimens of CD patients, presumably with longstanding disease and under immunomodulatory treatment [5]. Increased HEVs were found in inflamed colonic mucosa of UC patients and (to a lesser extent) in CD patients, compared to non-inflamed colonic mucosa of other IBD patients. However, no healthy controls were included, T cell maturation in the gut mucosa was not analysed and no serial biopsy samples at different time points and phases of disease within the same patients were analysed. Numbers of HEVs in patients with non-inflamed colonic mucosa have not been investigated during active disease and therefore it might concern a subgroup of patients without ectopic HEV formation similar to our HEV^{low} group. Our results expand upon this previous study, as we demonstrated differences in HEV density and TLOs in newly diagnosed, untreated IBD patients with active disease in correlation with different T cell subsets in the gut. Direct comparison with our presented results is difficult, as we included healthy controls and studied mucosal T cell maturation status and the presence of HEVs in relation to lymphoid follicles. We demonstrated increased extrafollicular HEVs formation in a subgroup of patients (HEV^{high}) in the early phase of disease. The lack of extrafollicular HEVs in the other subgroup (HEV^{low}) might be explained

by a different disease mechanism in these patients or the early phase of disease, assuming that formation of HEVs in inflamed mucosa is a characteristic of chronicity. Increased percentages of T_{EM} cells in HEV^{low} patients suggest predominant recruitment of T_{EM} cells into the inflamed gut. These T_{EM} cells, known to be $\alpha4\beta7^+$, might enter the inflamed gut by binding to MAdCAM-1 present on endothelial cell, in the absence of extrafollicular HEVs.

Lymphoid follicles in the gut were previously studied in established CD patients. In line with our findings in colonic samples of early IBD patients, they demonstrated the presence of submucosal *de-novo* lymphoid follicles in surgical ileal resection specimen of CD patients [17]. The authors interpreted these lymphoid follicles as being TLOs, based mainly on their location in the submucosa, muscularis propria and subserosa, but theoretically these follicles might still represent SLOs. However, no MECA-79 immunostaining with HEV quantification was performed [17]. Furthermore, the use of ileal resection specimen suggests a highly refractory patient group with longstanding disease. Potential differences between patients and associated mucosal T cell subsets were not investigated.

The mechanism of TLO development has not yet been clarified. Nevertheless, chemokines such as CXCL-13, CCL-19 and CCL-21 together with lymphotoxin $\alpha_1\beta_2$ ($LT\alpha_1\beta_2$, TNF family member) expressed on lymphoid tissue inducer cells (LTi) were shown to be required for lymphoid organogenesis [22]. $LT\alpha_1\beta_2$ is implicated in generation of follicular dendritic cells and HEVs out of stromal cells, while CCL-19, CCL-21 and CXCL-13 control the organization of the T- and B-cell follicular regions [12,22,25]. Previously, increased CXCL-13 expression has been observed in inflamed gut of UC patients [26], while research in transgenic mice demonstrated that CXCL-13 expression promotes B-cell recruitment and formation of lymphoid follicles [27]. Remarkably, we found increased serum CCL-19 and CXCL-13 in HEV^{high} patients. This suggests a systemic reflection of gut-localized formation of TLOs in these patients.

Recently, research in mice lacking LTi, it was demonstrated that lymphoid organogenesis was linked to the activation of stromal cells by TNF-α. This suggests that increased TNF-α expression during chronic inflammation might play an important role in TLO development in IBD [28]. Development of TLOs containing HEVs has been reported in other diseases with chronic inflammation and in patients with solid tumours. In patients with rheumatoid arthritis, TLOs were associated with an inferior therapeutic response to anti-TNF treatment and reversal of TLOs was a good marker of beneficial therapeutic response [29].

In our study, the higher number of colonic follicles found in the HEV^{high} group indicated that in this subgroup of IBD patients, de novo lymphoid follicles were being formed, representing TLOs. The patients in the HEV^{high} group not only had more follicles, they also exhibited more HEVs outside these follicles and this was correlated with a higher percentage of mucosal T_N+T_{CM} lymphocytes (known to be L-selectin$^+$ [18,19]) and a lower percentage of mucosal $T_{EM}+T_{EMRA}$ lymphocytes (known to be L-selectin$^-$ [18,19]). Remarkably, the patients in HEV^{low} group were comparable to healthy controls regarding these findings. HEVs express different

functional ligands (PNAd, MAdCAM-1) for L-selectin, facilitating extravasation and entry of T_N+T_{CM} lymphocytes into lymphoid organs [30]. Our findings suggest differences between IBD patients, both UC and CD, regarding TLO formation and recruitment of different lymphocyte subsets to the inflamed gut mucosa. These results further expand on our previous paper in newly diagnosed IBD patients with increased numbers of T_N and T_{CM} cells in the inflamed gut of a subgroup of patients [7]. Taken together, our results point out that increased homing of T_N and T_{CM} lymphocytes to non-lymphoid gut tissue in a subgroup of IBD patients might be facilitated by *de-novo* formation of extrafollicular HEVs and TLOs.

A limitation of the present study was that mucosal biopsy specimens precluded full examination of the deeper layers of the intestine wall (submucosa, muscularis propria and subserosa), which potentially contain HEVs and TLOs in IBD patients, particularly in CD. However, the strengths of our study were the prospective design, the sampling of untreated patients early in the course of IBD and the use of a standardized description of TLOs and HEVs in inflamed mucosa of patients. The clinical and therapeutic relevance of these findings in IBD patients remain to be elucidated; the presence of lymphoid follicles in initial biopsy specimens may, however, be predictive for colectomy due to drug refractory UC [31]. Furthermore, novel treatment strategies targeting T cell migration to the inflamed gut mucosa of IBD patients, such as anti-$\alpha4\beta7$ (vedolizumab (Entyvio®)) and anti-MAdCAM-1 monoclonal antibodies (PF-00547659) are emerging [32,33].

HEV^{low} patients with higher percentage of T_{EM} lymphocytes, suggesting increased recruitment of $\alpha4\beta7^+$ T cells, might theoretically benefit more and thus respond better to Vedolizumab treatment than HEV^{high} patients. Moreover, these findings may lead to further development of new treatment targets in IBD, designed to influence HEV and TLO formation by targeting the lymphotoxin pathway [34].

CONCLUSION

In conclusion, higher numbers of extrafollicular HEVs were demonstrated in IBD patients compared to healthy controls. An increased density of extrafollicular HEVs and TLOs in IBD patients was associated strongly with mucosal infiltration of T_N+T_{CM} lymphocytes, while a low density of HEVs correlated with higher numbers of mucosal T_{EM} cells. This suggests that the homing of T_N and T_{CM} lymphocytes to non-lymphoid gut tissue in IBD patients might be facilitated by extrafollicular HEVs and *de-novo* TLO formation within inflamed mucosa. Different 'T cell migration phenotypes' based on TLO formation in the early phase of IBD may allow for risk-stratification of patients and enable individualized treatment.

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4

Intestinal T cell profiling in Inflammatory Bowel Disease; linking T cell subsets to disease activity and disease course

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ABSTRACT

Introduction

A dysregulated intestinal T-cell response is presumed in patients with inflammatory bowel disease (IBD). In this longitudinal study, we investigated the changes in intestinal T lymphocyte subsets in IBD at first presentation and over time during endoscopic active or inactive disease and relate them to disease activity and outcome.

Methods

We included 129 newly diagnosed patients (87 Crohn´s disease (CD), 42 ulcerative colitis (UC)) and 19 healthy controls (HC). Follow-up biopsy specimens were analysed from 70 IBD patients. Immunophenotyping of specimens was performed by flow cytometry identifying lymphocyte subpopulations.

Results

IBD patients at diagnosis displayed higher percentages of CD4 T cells, Tregs and central memory T cells (T_{CM}) and with lower percentages of CD8 and CD103 T lymphocytes than HC. Follow-up specimens of patients with endoscopic inactive disease showed T-cell subset recovery comparable to HC. Endoscopic active disease at follow-up coincided with T-cell subsets similar to those at diagnosis.

In UC, lower baseline percentages of CD3 cells was associated with milder disease course without the need of an immunomodulator, whereas in CD, higher baseline percentages of CD4 and Tregs were associated with complicated disease course.

Conclusions

The intestinal T-cell infiltrate in IBD patients with active endoscopic disease is composed of increased percentages of $CD4^+$ T cells, Tregs, and T_{CM}, with lower percentages of $CD8^+$ T cells and $CD103^+$ T cells compared with HC and endoscopic inactive IBD. Baseline percentages of CD3, CD4 and Tregs were associated with disease outcome. Further research is needed to demonstrate the predictive value of these lymphocyte subsets.

INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are immune-mediated, inflammatory bowel diseases (IBD), with a chronic and relapsing character. Diagnosis is based on a combination of internationally accepted clinical, endoscopic, histological and radiological findings [1,2]. Incidence is increasing and disease burden is substantial due to the chronic nature and generally young age at onset of disease [3]. Disease presentation and course are heterogeneous and unpredictable. Approximately 50% of CD patients are expected to develop disease complications over time requiring aggressive medical therapy such as (biologic) immunomodulators or surgery [4,5]. In CD patients, clinical determinants associated with a complicated disease course are younger age (<40), perianal or stricturing disease, involvement of the upper gastrointestinal tract, penetrating disease, and smoking [6,7]. Colectomy is considered a solid endpoint for disease severity in UC, and is required in approximately 10% of patients in the first 10 years of disease. Extensive colitis, as well as severe systemic symptoms were shown to be predictors of colectomy [8 9,10].

Guidelines for the choice of treatment modality in IBD are still generic and historically based without much focus on the individual patient. Biomarkers that can predict disease course or response to therapy would help in focusing on the individual patient. Until now, results have been somewhat disappointing. Some efforts have been made in a study describing $CD8^+$ T cell transcriptional signatures associated with frequently relapsing disease in IBD patients [11]. Recently, a list of key research priorities regarding IBD treatment was compiled, including selection of patients for optimal treatment strategies [12]. Identifying biomarkers that relate to disease behaviour and response to therapy would help in this process. The first step in this process would be to identify potential biomarkers in IBD patients and to analyse the behaviour of these candidate biomarkers in patients over time. A dysregulated T lymphocyte response is often implicated as key mediator of chronic inflammation in IBD [13,14]. This is supported by the growing number of successful therapeutic approaches targeting T cells in IBD patients such as: thiopurines, inducing T cell apoptosis; anti-$\alpha 4\beta 7$, targeting the traffic of gut homing T cells and anti-IL12/23 p40, interfering with T-helper 1 (Th1) and Th17 lymphocytes [15].

Most research on T lymphocytes in IBD has focused on activated $CD4^+$ T effector lymphocytes (Th1, Th2, Th17 and regulatory T cells (Tregs)) [13]. The research on T lymphocytes in IBD was mainly based on cytokines detected in the mucosa of longstanding IBD patients and animal models. Instead of an overwhelming increase of effector memory T cells (T_{EM}), we previously identified newly diagnosed patients with increased percentages of naïve T lymphocytes (T_N) and central memory T lymphocytes (T_{CM}) in their inflamed gut mucosa[16]. Furthermore, it was shown that patients had higher percentages of Tregs and lower percentages of $CD103^+$ T cells at diagnosis, when compared with healthy controls. Differences in mucosal T cell subsets between IBD patients at diagnosis might have predictive value for response to different therapeutic agents. Our hypothesis is that intestinal mucosal T lymphocyte subsets may be

potential biomarkers for disease course in IBD. Therefore, the primary aim of this longitudinal study was to investigate the changes of intestinal mucosal T lymphocyte subsets in IBD patients at diagnosis and during follow-up in both presence and absence of endoscopic inflammation. A secondary aim was to study these subsets in relation to disease course over time.

MATERIAL AND METHODS

Study population

Patients suspected of IBD were prospectively enrolled at the outpatient clinic of the Crohn and Colitis Centre of Rijnstate Hospital Arnhem, The Netherlands. After standardised work-up including clinical, endoscopic, histopathological and, when applicable, radiological evaluation, all patients met the accepted international diagnostic criteria of CD or UC[1,2]. Patients were recruited before initial diagnostic ileocolonoscopy, enrolled after IBD was diagnosed, and subsequently followed in their clinical course. During the initial ileocolonoscopy, multiple biopsy specimens were taken for histopathological analysis and immunophenotyping by flow cytometry analysis. Lymphocyte subsets at baseline, for part of the IBD patients included in this study and healthy controls (HC) were previously described [16]. These groups were expanded with newly diagnosed patients and HC. Follow-up endoscopy was performed as part of standard care, taking additional biopsy specimens for immunophenotyping.

Healthy controls underwent ileocolonoscopy for polyp-surveillance and did not present endoscopic pathology. None of the healthy controls had any signs of IBD or other immune mediated diseases.

Classification and definitions

Endoscopic severity of the disease at baseline and follow-up was assessed in CD by the simple endoscopy score for CD (SES-CD; ranging from <4 to>19) and in UC by the Mayo score (ranging from 0 to 3) [17]. Inactive disease was defined as endoscopic remission (Mayo=0/ SES-CD<4). Active disease was defined as endoscopic disease activity (Mayo >0/ SES-CD>3). Active disease was further subdivided in mild (Mayo 1 or SES-CD 4-10), moderate (Mayo 2 or SES-CD 11-19) or severe (MAYO 3 or SES-CD>19). At baseline, all IBD patients were classified with active disease (Mayo >0/ SES-CD>3). At follow-up endoscopy, 36% of patients were classified as active and 56% as inactive disease.

Disease phenotype (location and behaviour) at baseline and at latest follow-up was assessed according to the Montreal classification [18]. Clinical disease activity at baseline and follow-up was assessed using the Harvey-Bradshaw index for CD and the Montreal classification for UC [18,19].

Overall use of and response to medication was recorded. In general, therapeutic management of IBD after diagnosis was done according to the step-up approach, as recommended by the

Dutch guidelines [20]. In UC patients, topical and/or oral 5-aminosalicylate (5-ASA) was initiated as induction therapy. In case of a severe pancolitis or when induction with 5-ASA treatment failed, patients were treated with topical or systemic steroids. When UC patients were steroid refractory, anti-tumour necrosis factor (TNF) therapy was started. When patients were steroid dependent, or had relapsing disease, a thiopurine was initiated and, in case of therapy failure or relapsing disease, anti-TNF treatment was initiated. Failure in either induction or maintenance of remission could lead to surgical escape therapy, i.e. colectomy. In CD patients, steroids were started as induction therapy. This was usually followed by the initiation of maintenance treatment (thiopurine derivates or methotrexate), especially in case of steroid-dependent disease. Anti-TNF therapy was initiated in case of steroid-refractory or steroid-dependent disease, or in case of extended perianal fistulising disease, usually in combination with a thiopurine derivate. If the disease did not respond to conventional treatment, resective surgery was considered. During relapse, escalation of maintenance treatment was considered.

We determined the highest treatment step in each patient, comparing 5-ASA, steroids, immunomodulator, anti-TNF and resective surgery as measures of severity of the disease. UC patients only needing 5-ASA treatment, and/or a short initial treatment with steroids, were classified as mild disease. Moderate UC was defined as the use of thiopurines after 5-ASA failure, and severe UC as the need for anti-TNF, vedolizumab or colectomy. CD patients with a mild disease course only needed 5-ASA, steroids, and/or an immunomodulator. Moderate CD was defined as the need for step up treatment above an immunomodulator (for instance anti-TNF therapy) without meeting the criteria of severe disease. Severe disease in CD was defined as the occurrence of penetrating or stricturing disease, perianal fistulizing disease with the need for anti-TNF initiation and/or surgical intervention, and CD-related resective surgery.

Tissue samples and cell preparation

Six intestinal biopsy specimens from IBD patients at baseline were obtained from the macroscopically most inflamed areas at primary diagnosis, before the initiation of any medical treatment. At follow-up endoscopy, six biopsy specimens were obtained from the macroscopically most inflamed areas or, when there was no inflammation, from the areas of inflammation at baseline. Regular histopathological analysis was performed at baseline and follow-up, as well as immunophenotyping of the mucosal lymphocyte populations.

In healthy controls, six biopsy specimens were randomly obtained from ileal and colonic areas. Regular histopathological examination confirmed the absence of inflammation, and immunophenotyping of the mucosal lymphocyte populations was performed.

Biopsy specimens meant for flow cytometry analysis were kept in phosphate-buffered saline solution at 2-8°C and processed within 8 h. Specimens were pooled and finely minced in Hanks'/1% bovine serum albumin using a 70-mm gaze and spatula followed by Ficoll density gradient centrifugation. The homogenate was resuspended, after washing, in 0.5 mL Hanks'/1% bovine serum albumin. The cell concentration of the mononuclear cell suspension

was estimated by microscopic counting with a KOVA glasstic slide (Hycor Biomedical, Penicuik, UK).

Flow cytometry

For flow cytometric analysis, 200 μL of the total cell suspension was used, irrespectively of the absolute cell numbers. Single cell suspensions of the biopsies were analysed using a FACS Canto (BD Biosciences). Intestinal lymphocyte subpopulations were defined as T cells ($CD3^+$) expressed as percentages of the whole lymphocyte population (CD45+/low side scatter). Mucosal T cells ($CD3^+CD103^+$), T-helper cells ($CD3^+CD4^+$) and cytotoxic T cells ($CD3^+CD8^+$) were expressed as percentages of the T cell population (CD3+/light scatter characteristics). Regulatory T cells (Tregs, $CD3^+CD4^+CD25^{high}FoxP3^+$) were expressed as percentages of the $CD3^+CD4^+$ population. The maturation state of T cells was assessed by using CD45RA and CD27 [21], defining naive T cells (T_N: $CD45RA^+CD27^+$), central memory T cells (T_{CM}: $CD45RA^-CD27^+$), effector memory T cells (T_{EM}: $CD45RA^-CD27^-$) and T effector memory cells re-expressing CD45RA (T_{EMRA}: $CD45RA^+CD27^-$) and expressed as percentages of the T cell ($CD3^+$) population.

Statistical analysis

All variables were tested for normality using the Shapiro-Wilk test. Categorical variables were expressed as numbers of patients and percentages and compared with chi-square or Fisher's exact test when necessary. Continuous variables were described as median and interquartile range (IQR) and compared with the Mann-Whitney U test. When more than two groups were compared, we used the Kruskal-Wallis test followed by the Dunn test when applicable. Paired data (baseline and follow-up percentages of lymphocyte subsets) were compared using the Wilcoxon matched-pairs signed-rank test. We performed hierarchical clustering analysis to discriminate the potential signature of inactive and active disease at follow-up using the software R[22]. Multivariate analyses were performed using binary logistic regression to identify independent predictors of disease course. Comparison of the initiation of thiopurine treatment in UC patients was performed using univariate and multivariate Cox-regression analysis, graphically represented by a Kaplan-Meier plot, resulting in a relative risk (RR) with corresponding confidence interval (CI). SPSS statistics for Windows (version 22.0; IBM Corp, Armonk, NY, USA) and GraphPad Prism (GraphPad Software version 7.0, La Jolla, CA, USA) were used to analyse data. Statistical significance was defined as a *P*-value lower than 0.05.

Ethical considerations

Written informed consent was obtained from each participating patient before any study-related procedure was performed. The procedures were performed in accordance with the Declaration of Helsinki. The regional medical ethics committee approved the study protocol (NL28761.091.09).

RESULTS

Patient characteristics

This study included 87 CD patients, 42 UC patients and 19 HC. Baseline characteristics can be found in table 1. IBD patients were significantly younger than HC (median 28 years (IQR=21-40) vs. 40 years (IQR=32-57), *P*=0.0001). There were more females in the HC group (89% vs. 66%, *P*=0.03). The median follow-up duration after initial diagnosis was 32 months (IQR 18-66) for CD patients and was significantly longer compared with UC patients (23 months (IQR 13-38), *P*=0.004). Endoscopic disease severity, categorised as mild, moderate, or severe disease, was evenly distributed in both CD and UC. CD patients had longer symptom duration before the establishment of primary diagnosis (*P*=0.002), and a larger proportion were smokers (*P*=0.002) and had a higher baseline C- reactive protein (CRP) (*P*=0.0001) than UC patients.

Table 1. Baseline patient characteristics.

	CD (n= 87)	UC (n=42)	*P*-value CD vs UC	HC (n=19)
Patient characteristics				
Median age at diagnosis in years (IQR)	26 (21-41)	28 (22-38)	0.61	40 (32-57)
Female/male	58/29	27/15	0.47	17/2
Duration of symptoms before diagnosis, n (%)			0.002	
- 0-3 months	- 27 (31%)	- 23 (55%)		
- 3-6 months	- 20 (23%)	- 13 (31%)		
- >6 months	- 40 (46%)	- 6 (14%)		
First degree relative with IBD, n (%)	12 (14%)	11 (26%)	0.12	0 (0%)
Smoking at diagnosis, n (%)			0.002	
- Current	- 38 (44%)	- 5 (12%)		- 6 (32%)
- Former	- 8 (9%)	- 6 (14%)		- 2 (11%)
- Never	- 41 (47%)	- 31 (74%)		- 11 (58%)
Laboratory values at diagnosis, n, median, (IQR)				N=14, 3 (1-8) N=5, 2 (0-7)
CRP	N=78, 19 (9-56)	N=35, 5 (1-17)	0.0001	
Faecal calprotectin	N=41, 488 (251-1237)	N=25, 396 (239-825)	0.54	
Median follow-up in months (IQR)	32 (18-66)	23 (13-38)	0.004	

Table 1. continued

	CD (n= 87)	UC (n=42)	*P*-value CD vs UC	HC (n=19)
Disease characteristics				
Harvey Bradshaw Index, median (IQR)	8 (6-12)			
- Remission (<5)	8 (9%)			
- Mild (5-7)	30 (34%)			
- Moderate (8-16)	39 (45%)			
- Severe (>16)	10 (12%)			
SES-CD (0-56), median (IQR)	12 (8-22)			
- Remission (<4)	- 0 (0%)			
- Mild (4-10)	- 34 (39%)			
- Moderate (11-19)	- 27 (31%)			
- Severe (>19)	- 26 (30%)			
Disease location (CD) at diagnosis, n (%)	- 30 (34%)			
- L1 (ileal)	- 17 (20%)			
- L2 (colonic)	- 40 (46%)			
- L3 (ileocolonic)	- 23 (26%)			
- L4 (proximal of ileum)				
Disease behaviour (CD) at diagnosis, n (%)	- 68 (78%)			
	- 15 (17%)			
- B1 (non-stricturing, non-penetrating)	- 4 (5%)			
- B2 (stricturing)	- 12 (14%)			
- B3 (penetrating)				
- P (Perianal disease)				
Montreal classification of clinical severity at diagnosis, n (%)				
- S0 (remission)		- 0 (0%)		
- S1 (mild)		- 13 (31%)		
- S2 (moderate)		- 16 (38%)		
- S3 (severe)		- 13 (31%)		
Endoscopic MAYO-score at diagnosis, n (%)				
- Mayo 0		- 0 (0%)		
- Mayo 1		- 9 (21%)		
- Mayo 2		- 24 (58%)		
- Mayo 3		- 9 (21%)		

Table 1. continued

	CD (n= 87)	UC (n=42)	*P*-value CD vs UC	HC (n=19)
Highest treatment to reach initial remission			0.0001	
- No treatment	- 2 (2%)	- 0 (0%)		
- 5ASA	- 4 (5%)	- 23 (55%)		
- Steroids	- 17 (19%)	- 10 (24%)		
- Immunomodulator	- 46 (53%)	- 6 (14%)		
- Biological	- 12 (14%)	- 1 (2%)		
- Surgery	- 6 (7%)	- 2 (5%)		

Lymphocyte distribution at baseline in IBD patients compared with healthy controls

To determine if lymphocyte subsets' distribution in the inflamed gut was influenced by biopsy location, we compared lymphocyte subset percentages from CD patients with ileal location (n=30), biopsy specimens being taken only from the inflamed ileum, with those with colonic location (n=17), biopsy specimens being taken only from the inflamed colon. There were no statistical differences for any of the analysed lymphocyte subsets between ileal and colonic location in CD (supplementary table 1). Therefore, we analysed the ileal and colonic lymphocyte subsets from CD patients together. IBD patients showed a distinctive composition of T lymphocyte subsets in their inflamed intestine when compared with HC (table 2). They had higher percentages of $CD4^+$ T cells with lower percentages of $CD8^+$ T cells whereas HC had higher percentages of mucosal $CD103^+$ T cells and lower percentages of Tregs. When examining T cell maturation in patients and controls, IBD patients had higher percentages of T_{CM} cells. The difference in relative amounts of T lymphocyte subsets compared with HC was most pronounced in UC patients. They had higher percentages of $CD4^+$ T cells and percentages of T_{CM} cells as well as lower percentages of $CD8^+$ T cells and percentages of $CD103^+$ T cells, than CD patients.

Table 2. Baseline intestinal lymphocyte populations in healthy controls (HC), Crohn's disease (CD) and ulcerative colitis (UC). Medians (interquartile range) are reported.

	HC (n=19)	CD (n=87)	UC (n=42)	*P*-value[ab]	*P*-value[ac]	*P*-value[d]
$CD3^+$ as % of lymphocytes	66 (56-69)	53 (45-64)	58 (48-69)	0.021	0.28	0.17
$CD3^+CD4^+$ as % of T cells	48 (31-76)	67 (53-75)	73 (64-82)	0.005	0.0001	0.004
$CD3^+CD8^+$ as % of T cells	45 (24-58)	28 (22-36)	22 (16-29)	0.003	0.0001	0.003
$CD3^+CD103^+$ as % of T cells	43 (23-66)	17 (9-34)	12 (6-21)	0.001	0.0001	0.009
T_N* as % of T cells	9 (3-25)	17 (9-35)	19 (11-30)	0.33	0.27	0.97
T_{CM}*as % of T cells	21 (11-27)	33 (24-45)	50 (38-57)	0.032	0.002	0.0001
T_{EM}* as % of T cells	41 (24-52)	26 (10-45)	20 (12-29)	0.30	0.008	0.23
Treg* as % of T cells	4 (2-5)	11 (8-16)	10 (8-16)	0.001	0.004	0.80

*Subsets were defined as described in methods: T_N by $CD3^+CD27^+CD45RA^+$, T_{CM} by $CD3^+CD27^+$ $CD45RA^-$, T_{EM} by $CD3^+CD27^-CD45RA^-$ and Treg by $CD3^+CD4^+CD25^+Foxp3^+$

a. corrected for age

b. comparing CD and HC

c. comparing UC and HC

d. comparing CD and UC

Development of lymphocyte subsets during follow-up in IBD patients

Seventy patients (54% of all IBD patients) underwent follow-up endoscopy with consecutive lymphocyte subsets analysis. From these patients, 25 had active endoscopic disease at the time of the follow-up endoscopy (36%), and 39 had inactive endoscopic disease (56%). Discrepancy of endoscopic disease activity between ileum and colon was observed in six CD patients (8%), with active disease in one bowel segment and inactive disease in the other segment, although these patients had ileocolonic disease at baseline. These patients were left out of the analysis on the change of the lymphocyte subsets between baseline and follow-up.

When compared with baseline, patients with inactive endoscopic disease at follow-up had decreased percentages of $CD4^+$ T cells (fold change (FC) =0.78, *P*=0.001) and increased percentages of $CD8^+$ T cells (FC=1.46, *P*=0.0001,) (figure 1 and supplementary table 2). They also presented increased percentages of $CD103^+$ T cells (FC=2.43, *P*=0.0001), decreased percentages of Tregs (FC=0.38, *p*=0.004), and a shift towards more T_{EM} (FC 1.48, *P*=0.0001) leading to lower percentages of T_N and percentages of T_{CM} (FC=0.45 and FC=0.55; *P*=0.002 and *P*=0.0001, respectively).

Patients with active endoscopic disease at follow-up showed an even further decrease of percentages of $CD8^+$ cells compared with their baseline percentages (FC=0.76, *P*=0.016). All other T lymphocyte subsets did not differ statistically from baseline in patients with active

endoscopic disease. Subdividing UC and CD patients gave similar results to those among the whole group (figure 1, supplementary table 2).

For baseline intestinal lymphocyte populations of the endoscopic inactive and active disease group at follow-up see supplementary tables 5-7, percentages at baseline were comparable among both groups.

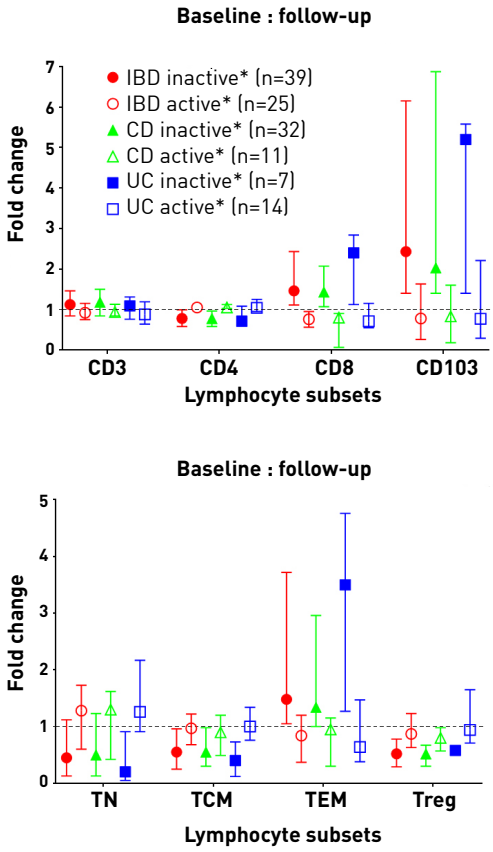

Figure 1. Fold change of different lymphocyte subsets in inflammatory bowel disease (IBD) patients at follow-up endoscopy with either endoscopic active or inactive disease when compared to their baseline values (all with active disease) by Wilcoxon matched-pairs signed-rank test.

IBD patients with inactive endoscopic disease at follow-up endoscopy had a statistical significant decrease compared to baseline percentages of CD4 T cells, naïve T cells (TN), central memory T cells (TCM) and regulatory T cells (Treg) with a significant increase of %CD8 T cells, CD103 T cells and effector memory T cells (TEM). In patients with active endoscopic disease at follow-up, T lymphocyte subsets did not differ significantly from baseline percentages, except for an even further decrease of the CD8%. When subdividing ulcerative colitis (UC) and Crohn's disease (CD) patients the same patterns were found.

*inactive (MAYO=0/SES-CD<4) or active (MAYO>0/SES-CD>3) disease at follow-up endoscopy

We additionally performed a hierarchical cluster analysis to compare the lymphocyte subsets signature of patients with active endoscopic disease with those with inactive endoscopic disease at follow-up (figure 2). We found that patients with active endoscopic disease had higher percentages of $CD4^+$ T cells, lower percentages of $CD8^+$ T cells, lower percentages of $CD103^+$ T cells, higher percentages of Tregs, higher percentages of T_{CM} and lower percentages of T_{EM} (all: P=0.0001) and higher percentages of T_N (P=0.001) than patients with inactive endoscopic disease (figure 3 and 4, table 3). Comparing the different grades (mild vs moderate vs severe) of endoscopic disease severity in active disease did not show mutual significant differences. The same results were found when subdividing UC and CD (supplementary tables 3 and 4).

The percentages of T lymphocyte subsets in patients without endoscopic disease activity recovered to levels comparable to HC (figure 3 and 4, table 3). Medical treatment at the moment of the follow-up endoscopy was very different between patients. No statistical differences were found for lymphocyte subsets percentages when adjusting for the different regimens. However, no definite conclusions can be drawn about the effect of treatment modality on lymphocyte subsets due to the small sample size per treatment regimen.

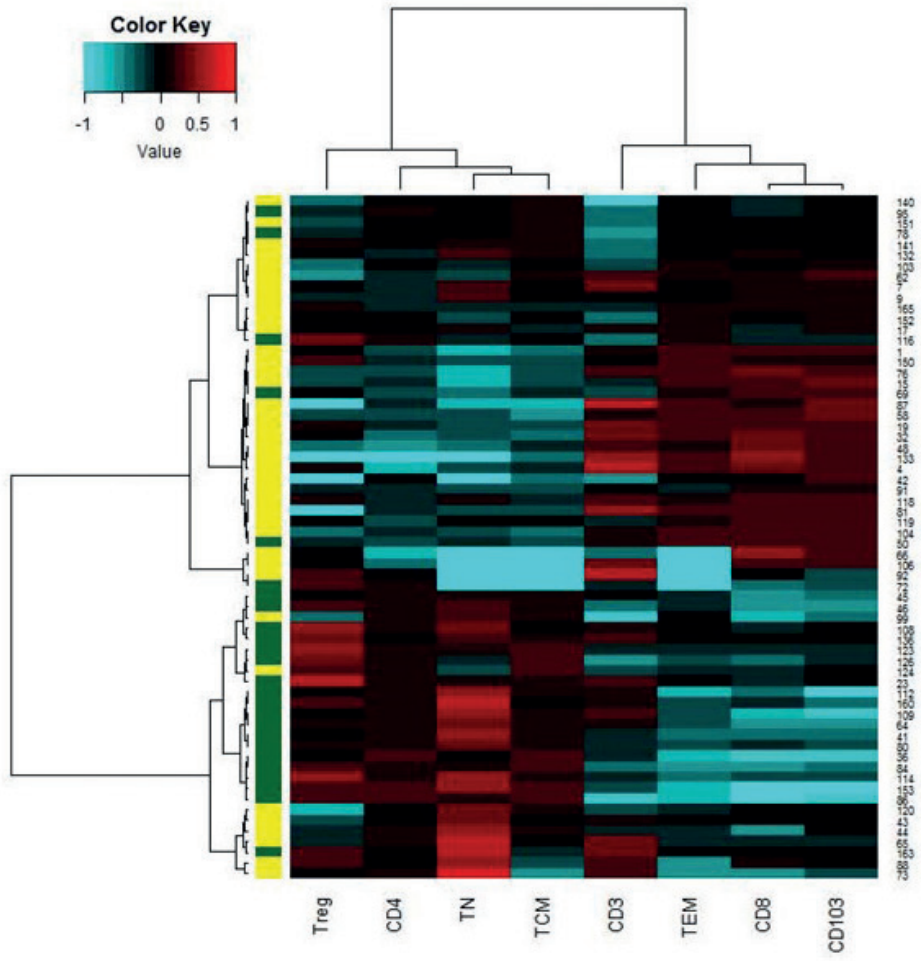

Figure 2. Hierarchical clustering analysis comparing IBD patients at follow-up with active endoscopic (green) and inactive endoscopic disease (yellow).

TN = naïve T cells, TCM = central memory T cells, TEM = effector memory T cells, Treg = regulatory T cells.

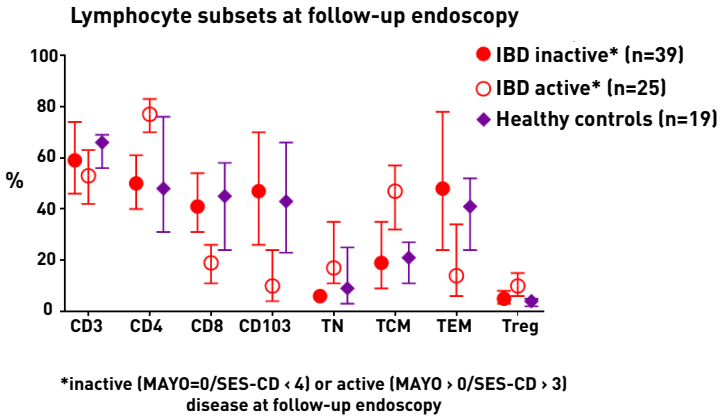

Figure 3. Intestinal lymphocyte subsets at follow-up endoscopy in inflammatory bowel disease (IBD) patients with endoscopic active and -inactive disease and healthy controls.

The T cell subsets from patients with active endoscopic disease showed a distinct pattern from healthy controls and patients with inactive endoscopic disease, while T cell subsets from patients with inactive endoscopic disease approached those of healthy controls.
TN= naïve T cells, TCM= central memory T cells, TEM = effector memory T cells, Treg = regulatory T cells.
* inactive (MAYO=0/SES-CD<4) or active (MAYO>0/SES-CD>3) disease at follow-up endoscopy

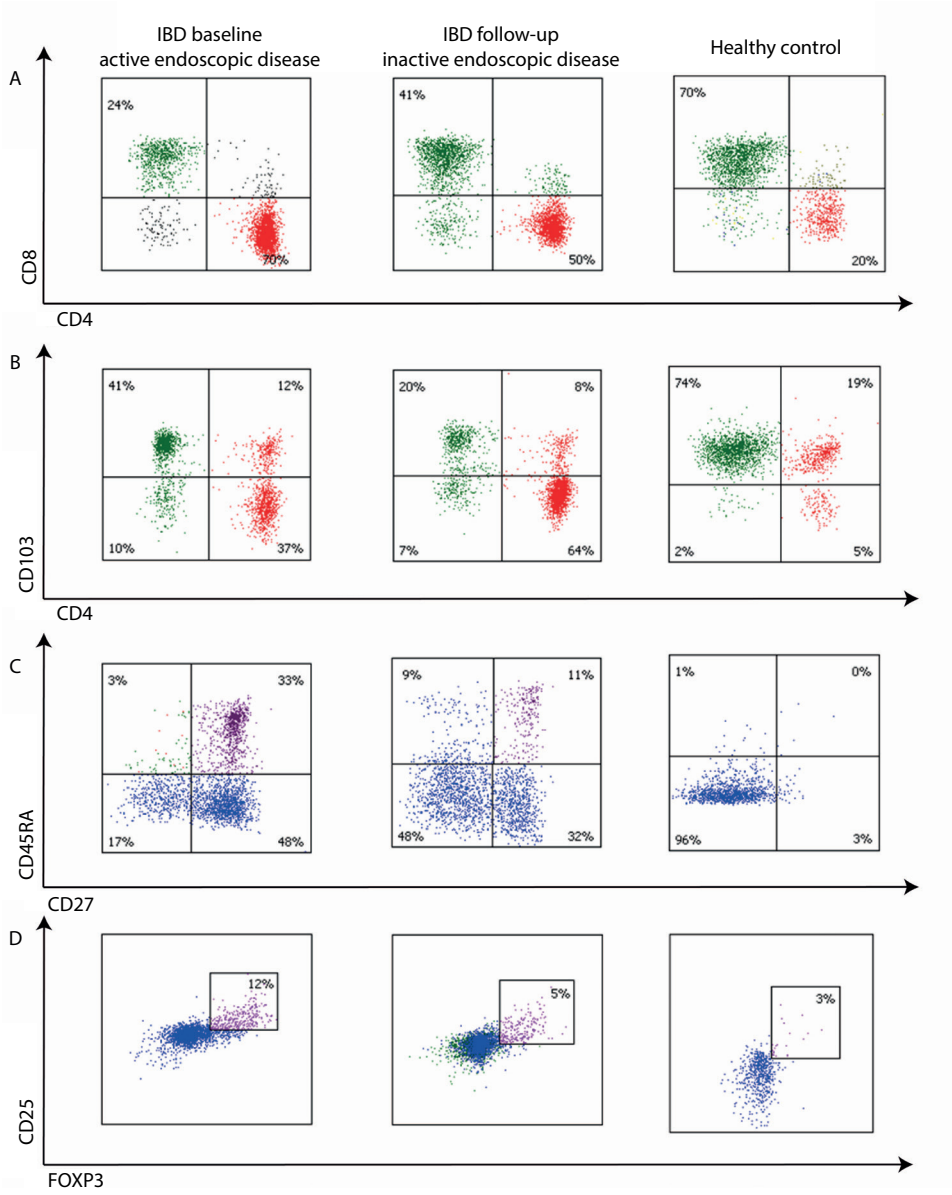

Figure 4. Representative flow cytometric dot plots showing (from left to right) a patient with ulcerative colitis at diagnosis with active endoscopic disease and the same patient at follow-up endoscopy with inactive endoscopic disease next to a healthy control.

A. shows CD4/CD8 gated in the $CD3^+$ population, B. shows $CD103^+$ cells within the $CD3^+$ population, C. shows T cell maturation based on CD27 and CD45RA within the $CD3^+$ population with naïve T cells ($CD27^+CD45RA^+$), central memory T cells ($CD27^+CD45RA^-$), effector memory T cells ($CD27^-CD45RA^-$) and effector memory T cells re-expressing CD45RA ($CD27^-CD45RA^+$), D. shows regulatory T cells ($CD25^+FoxP3^+$) within the $CD3^+CD4^+$ population.

Table 3. Intestinal lymphocyte populations at follow-up endoscopy from IBD patients with either endoscopic active (MAYO>0/ SES-CD>3) or inactive (MAYO=0/ SES-CD<4) disease and healthy controls (HC). Medians (interquartile range) are reported.

	IBD: endoscopic inactive disease (n=39)	IBD: endoscopic active disease (n=25)	*P*-value[a]	HC (n=19)	*P*-value[bc]	*P*-value[cd]
FU duration from diagnosis until follow-up endoscopy in months, median (IQR)	24 (13-47)	22 (11-39)	0.27	-	-	-
$CD3^+$ as % of lymphocytes	59 (46-74)	53 (42-63)	0.09	66 (56-69)	0.41	0.013
$CD3^+CD4^+$ as % of T cells	50 (40-61)	77 (70-83)	0.0001	48 (31-76)	0.86	0.002
$CD3^+CD8^+$ as % of T cells	41 (31-54)	19 (11-26)	0.0001	45 (24-58)	0.71	0.002
$CD3^+CD103^+$ as % of T cells	47 (26-70)	10 (4-24)	0.0001	43 (23-66)	0.62	0.004
T_N* as % of T cells	6 (4-18)	17 (11-35)	0.001	9 (3-25)	0.23	0.34
T_{CM}*as % of T cells	19 (9-35)	47 (32-57)	0.0001	21 (11-27)	0.86	0.006
T_{EM}* as % of T cells	48 (24-78)	14 (6-34)	0.0001	41 (24-52)	0.11	0.043
Treg* as % of T cells	5 (3-8)	10 (6-15)	0.0001	4 (2-5)	0.19	0.004

*Subsets were defined as described in methods: T_N by $CD3^+CD27^+CD45RA^+$, T_{CM} by $CD3^+CD27^+$ $CD45RA^-$, T_{EM} by $CD3^+CD27^-CD45RA^-$ and Treg by $CD3^+CD4^+CD25^+Foxp3^+$

a. Comparing endoscopic active and inactive IBD
b. Comparing endoscopic inactive IBD and HC
c. Corrected for age
d. Comparing endoscopic active IBD and HC

T cell maturation profiles at baseline and during follow-up

Previously, we distinguished four T-cell maturation profiles in the intestinal mucosa of IBD patients (figure 5) [16]. At baseline, 71% of UC patients had a B profile with predominantly T_{CM}, whereas CD patients were more heterogeneous (B in 34%, C in 30%, A in 17% and D in 16%) and HC had mainly a C or D profile (47% and 37% respectively, figure 5). T cell maturation profiles were not associated with disease phenotypes or course.

In patients with IBD with active endoscopic disease at follow-up endoscopy, 89% of patients with a B profile at baseline retained this profile at follow-up. Among patients with a C or D profile at baseline, 50% changed to an A or B profile when there was disease activity at follow-up endoscopy.

On the other hand, in IBD patients with inactive endoscopic disease at follow-up endoscopy, the A and B profile changed to C or D in 79% of patients. The change of profile from baseline to

follow up in patients with inactive disease at follow-up endoscopy was statistically significant (*P*=0.014). Patients with C or D profile at baseline maintained this profile largely when there was inactive endoscopic disease at follow-up (86%).

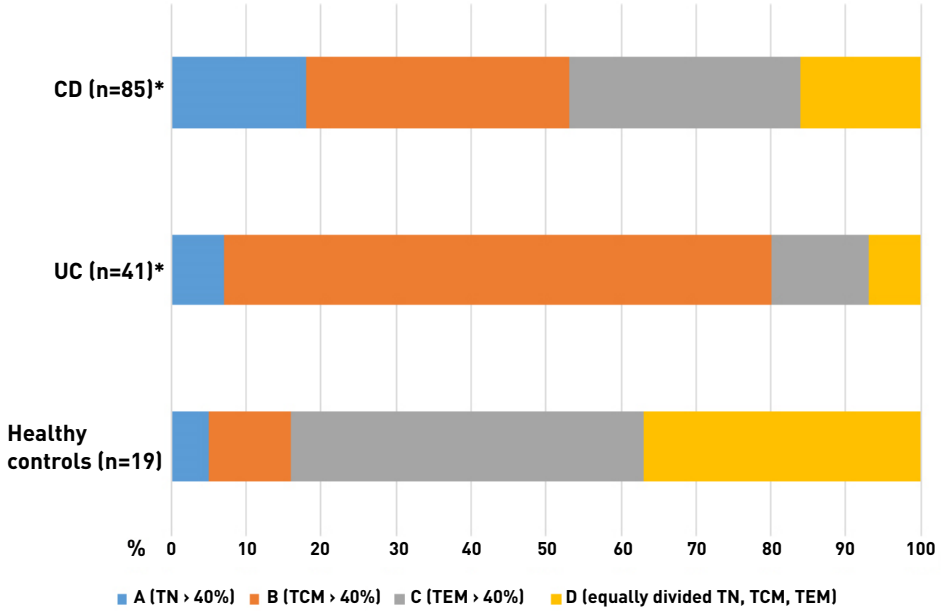

Figure 5. Different intestinal T cell maturation profiles at baseline in healthy controls and IBD patients.

*in two IBD patients (1 UC and 1 CD) the maturation profiles were not determined (technical reasons)

Predicting disease course in UC

We determined the association between baseline lymphocyte subsets and disease course in UC patients with a follow-up duration of longer than one year (n=33, 79% of all UC patients). Of these patients, 27 had a disease exacerbation during follow-up and six patients retained remission. Their follow-up duration was comparable (respectively a median of 26 months (IQR 19-54) and 38 months (20-56), *P*=0.80). During their follow-up, 13 UC patients used 5-ASA as highest treatment strategy (39%), three patients needed steroids as highest step (9%), 12 patients needed an immunomodulator (36%), two patients used anti-TNF as highest treatment step (6%), and three UC patients ultimately needed resective surgery (9%). Follow-up duration of these groups was not different (*P*=0.10). UC patients with 5-ASA as highest treatment strategy (n=13) had lower baseline percentages of $CD3^+$ cells (median 48% (43-63), *P*=0.03) than patients in need of step-up treatment (n=20, median 62% (50-71)). Patients with an immunomodulator as highest treatment strategy (n=12) had higher baseline percentages of

$CD3^+$ cells (median 65% (57-78), *P*=0.005) than patients who only needed 5-ASA and/or steroids (n=16, 49% (44-66)).
There were no associations between the percentages of lymphocyte subsets at baseline and the following disease outcomes: surgery, biologics, relapsing disease, or future disease severity.

Predicting disease course in CD

We determined the association between lymphocyte subsets at baseline and disease course in CD patients with a follow-up duration of longer than one year (n=77, 90% of all CD patients). Disease exacerbation occurred in 43 CD patients (56%) and 34 patients (44%) retained remission, with comparable follow-up duration between these groups (*P*=0.10). From this group, three CD patients (4%) only used 5-ASA during follow-up, nine patients (12%) only needed steroids, 34 patients (44%) used an immunomodulator as highest treatment step, 22 patients (29%) used anti-TNF as highest treatment strategy and nine CD patients (12%) ultimately needed resective surgery. Follow-up duration of the groups was generally comparable, except between the surgery and steroid groups (*P*=0.03). Compared with uncomplicated inflammatory disease (B1, n=60), future stricturing or penetrating disease (B2/B3, n=17) was associated with higher baseline percentages of $CD4^+$ cells (median 74% (IQR=65-79) vs. 65% (IQR=52-73), *P*=0.02) and higher baseline percentages of Tregs (median 15% (IQR=9-23) vs. 11% (IQR=7-15) *P*=0.04). Follow-up duration was comparable between these two groups (*P*=0.49).
Abdominal surgery was associated with higher baseline percentages of Tregs compared with patients who did not need surgery (median percentages of Tregs 15% (IQR=14-19) vs. 10% (IQR=7-15), *P*=0.014). However, after correction for follow-up duration this was not significant anymore (*P*=0.051). Steroids as highest treatment modality (n=9) were associated with lower baseline percentages of Tregs (median 8% (IQR=5-10), *P*=0.014) compared with patients needing for step-up treatment (n=65, median 11% (IQR=8-16)). This remained significant after correction for follow-up duration (*P*=0.033).

DISCUSSION

In the present study, marked differences were demonstrated in intestinal T lymphocyte subset composition of IBD patients with active endoscopic disease when compared with HC and patients in with inactive endoscopic disease. IBD patients at diagnosis displayed higher percentages of CD4, Tregs and T_{CM} with lower percentages of CD8 and CD103 T lymphocytes. Patients with active endoscopic disease at follow-up analysis maintained the same compositions of T cell subsets as at the moment of diagnosis with even lower percentages of CD8 T lymphocytes. This confirms consistency of T cell subset composition in time. Follow-up biopsy specimens of IBD patients with inactive endoscopic disease showed recovery of these subsets to a T lymphocyte composition, comparable to HC. Lymphocyte subset percentages were associated with

endoscopic disease activity. Furthermore, in UC lower baseline percentages of CD3 cells were associated with a milder disease course without the need of step-up to an immunomodulator, whereas in CD higher baseline percentages of CD4 and higher percentages of Tregs were associated with a more complicated disease course.

There were no differences found between ileum and colon regarding lymphocyte subsets: the composition of subsets seems more disease related than location related. This is endorsed by the association of the different lymphocyte subsets with endoscopic disease activity. Differences in lymphocyte subsets were more pronounced in UC than in CD patients. This might be explained by the fact that UC is characterised by more continuous inflammation conveyed to the mucosa and submucosa with high chances that biopsy specimens from these patients are abundantly inflamed, whereas in CD biopsy samples might have been taken from the edge of an ulcer with partly uninflamed tissue.

Intestinal inflammation in IBD has been attributed to CD4 subsets based on animal models and cytokine expression in the gut mucosa [23]. However, auto-reactive CD8 cells have also been suggested as instigators of the disease by destruction of epithelial cells after which exposure to luminal antigens may attract and expand the CD4 lineage exacerbating inflammation [24]. The imbalance that we found of increased CD4 with decreased CD8 frequencies in active endoscopic IBD at baseline and follow-up seems to represent a CD4 expansion over CD8, but cannot provide further information on the real instigators of disease. The imbalance was normalised in the endoscopically inactive mucosa of IBD patients in remission, approaching comparable frequencies to HC. The percentages CD8 and CD4 T cells that we found in the intestines of controls was consistent with a previous study in organ donors [25]. In our study in CD patients, higher baseline percentages of CD4 were associated with the development of strictures or penetrating disease. This could represent increased expansion of (proinflammatory) CD4 lymphocytes accompanied by Treg expansion, in an (unsuccessful) attempt to diminish inflammation, leading to disease complications by unknown mechanisms that need to be investigated further.

Tregs are involved in the maintenance of intestinal homeostasis by suppressing abnormal responses to enteric antigens by direct interaction with other cells and through secretion of anti-inflammatory cytokines like TGF-beta and IL-10 [26]. In this study, we showed that IBD patients have increased percentages of Tregs at diagnosis (always with active endoscopic disease) and when there is active endoscopic disease during follow-up, whereas percentages decrease significantly in the same patients at times of inactive endoscopic disease. Furthermore, in CD patients, higher baseline percentages of Tregs were associated with a more complicated disease course with strictures or penetration and a trend was observed towards more abdominal surgeries. Lower baseline percentages of Tregs in CD patients was associated with a milder

course with steroids as highest treatment step. It is tempting to speculate about a hampered function of Tregs in CD to be compensated by numbers. Increased mucosal Treg frequencies have been shown in active IBD before [27]. This could represent active recruitment and local expansion of these cells, attempting to suppress inflammation. However, this expansion is apparently not sufficient to control inflammation, possible due to the still too small number of cells. Another explanation might be that their function is impaired by upregulation of Smad7 in IBD patients, which inhibits TGF-beta signalling, thereby impairing the suppression of T cell activation and proliferation but possibly also the differentiation of naïve T cells into Tregs [28,29]. Finally, there is evidence that transformation of Tregs into proinflammatory Th17 cells could also contribute to uncontrolled inflammation [26].

We demonstrated that IBD patients with active endoscopic disease had lower percentages of $CD103^+$ T lymphocytes compared to HC, and a significant increase of percentages of $CD103^+$ T lymphocytes in patients with endoscopic inactive disease at follow-up endoscopy. CD103 is the integrin αE of αEβ7 expressed on several immune cell subsets and its expression is thought to contribute to the retention of T lymphocytes within the mucosa. Our findings suggest a less prominent role for $CD103^+$ T cells as pathogenic factor in IBD patients than previously assumed, as it was thought to be upregulated in active endoscopic disease [30-32]. Therefore, it would be very interesting to study the $CD103^+$ cell subpopulations in more detail with the upcoming development of anti-β7 treatment. The function of the $\alpha E\beta 7^+$ T lymphocytes needs to be further investigated. αEβ7 is present on tissue-resident-memory T cells (T_{RM}, mainly on $CD8^+$ T cells) with an immunosurveillance and protective function[33]. Recently, lung-derived $CD103^+T_{RM}$ cells were shown to possess a gene-expression programme associated with the inhibition of T cell activation, which might play a role in preventing excessive immunoreactivity [34]. $\alpha E\beta 7^+$ T lymphocytes might even be necessary to achieve or maintain remission in patients and a definitive pathogenic role of these cells in IBD has not yet been shown. Thus, from a mechanistic point of view, its blockade remains questionable [35].

CONCLUSION

The intestinal T cell infiltrate in IBD patients with active endoscopic disease is composed of increased $CD4^+$ T cells, Tregs and T_{CM}, with lower percentages of $CD8^+$ T cells and $CD103^+$ T cells compared with HC and endoscopically inactive IBD. After patients entered endoscopic remission, the lymphocyte subset composition of the gut recovered to percentages comparable with HC. Baseline percentages of CD3, CD4 and Tregs were associated with disease outcome in this study. Further research is needed to demonstrate the predictive value of these lymphocyte subsets. Knowledge of intestinal immune subsets at baseline and during follow-up could guide the use of new drugs and should be incorporated in the early phases of drug development to identify predictors of response.

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SUPPLEMENTARY TABLES

Supplementary table 1. Baseline lymphocyte subsets in Crohn's disease (CD) patients with solitary ileal and colonic disease. Median (interquartile range IQR) are reported.

	CD solitary ileal disease	CD solitary colonic disease	*P*-value
Number of patients	30	17	
$CD3^+$ as % of lymphocytes	51 (39-66)	62 (48-68)	0.14
$CD3^+CD4^+$ as % of T cells	70 (52-78)	67 (58-72)	0.63
$CD3^+CD8^+$ as % of T cells	26 (20-36)	28 (23-32)	0.62
$CD3^+CD103^+$ as % of T cells	15 (8-36)	15 (9-34)	0.56
T_N* as % of T cells	20 (9-33)	26 (12-44)	0.44
T_{CM}*as % of T cells	41 (25-48)	38 (25-44)	0.37
T_{EM}* as % of T cells	27 (10-43)	25 (7-47)	0.94
Treg* as % of T cells	12 (6-17)	11 (9-13)	0.67

*Subsets were defined as described in methods: T_N by $CD3^+CD27^+CD45RA^+$, T_{CM} by $CD3^+CD27^+CD45RA^-$, T_{EM} by $CD3^+CD27^-CD45RA^-$ and Treg by $CD3^+CD4^+CD25^+Foxp3^+$

Supplementary table 2. Fold change (FC) of different lymphocyte populations at follow-up endoscopy with active or inactive endoscopic disease when compared to their baseline values (all active disease), looking at al IBD patients (n=64) and subdividing CD (n=43) and UC (n=21).

	%CD3	%CD4	%CD8	%CD103	%TN	%TCM	%TEM	%Treg
IBD inactive (n=39)	1.12 (0.84-1.46)	0.78 (0.58-0.99)	1.46 (1.11-2.43)	2.43 (1.40-6.15)	0.45 (0.13-1.12)	0.55 (0.25-0.96)	1.48 (1.05-3.72)	0.52 (0.29-0.78)
P-value*	0.047	0.001	0.0001	0.0001	0.002	0.0001	0.0001	0.0001
IBD active (n=25)	0.92 (0.75-1.15)	1.05 (0.98-1.13)	0.76 (0.56-0.95)	0.78 (0.26-1.63)	1.28 (0.60-1.73)	0.97 (0.68-1.22)	0.84 (0.37-1.20)	0.87 (0.63-1.23)
P-value*	0.367	0.050	0.016	0.618	0.426	0.648	0.236	0.126
CD inactive (n=32)	1.18 (0.84-1.50)	0.78 (0.58-0.96)	1.43 (1.07-2.07)	2.03 (1.40-6.87)	0.50 (0.13-1.23)	0.55 (0.30-0.98)	1.34 (1.00-2.96)	0.52 (0.30-0.67)
P-value*	0.071	0.003	0.001	0.0001	0.020	0.001	0.003	0.0001
CD active (n=11)	0.94 (0.84-1.13)	1.05 (1.03-1.12)	0.80 (0.63-0.90)	0.83 (0.18-1.60)	1.30 (0.42-1.62)	0.90 (0.49-1.20)	0.95 (0.30-1.15)	0.80 (0.57-0.98)
P-value*	0.859	0.056	0.041	0.824	0.789	0.423	0.508	0.012
UC inactive (n=7)	1.09 (0.76-1.31)	0.71 (0.61-1.08)	2.40 (1.12-2.84)	5.20 (1.40-5.58)	0.20 (0.05-0.91)	0.40 (0.12-0.73)	3.50 (1.27-4.76)	0.58 (0.14-1.63)
P-value*	0.398	0.236	0.128	0.028	0.043	0.237	0.028	0.293
UC active (n=14)	0.88 (0.64-1.19)	1.04 (0.95-1.25)	0.71 (0.55-1.15)	0.77 (0.29-2.21)	1.26 (0.91-2.17)	1.00 (0.76-1.34)	0.64 (0.38-1.47)	0.94 (0.71-1.65)
P-value*	0.220	0.209	0.133	0.396	0.099	0.969	0.346	0.834

* Wilcoxon matched-pairs signed-rank test, comparing follow-up lymphocyte subsets to baseline lymphocyte subsets

Supplementary table 3. Intestinal lymphocyte populations at follow-up endoscopy from CD patients with endoscopic inactive CD (SES-CD<4) or endoscopic active CD (SES-CD>3). Medians (interquartile range) are reported.

	Inactive CD (n=32)	Active CD (n=11)	*P*-value IBD patients
FU duration in months	36 (13-53)	24 (5-40)	0.138
$CD3^+$ as % of lymphocytes	63 (46-73)	59 (49-66)	0.651
$CD3^+CD4^+$ as % of T cells	49 (40-61)	73 (55-82)	0.015
$CD3^+CD8^+$ as % of T cells	41 (31-53)	22 (16-32)	0.009
$CD3^+CD103^+$ as % of T cells	48 (27-72)	23 (6-32)	0.004
T_N* as % of T cells	7 (4-19)	16 (7-43)	0.180
T_{CM}*as % of T cells	19 (9-35)	39 (19-47)	0.064
T_{EM}* as % of T cells	45 (25-77)	29 (3-43)	0.056
Treg* as % of T cells	4 (3-7)	8 (6-13)	0.003

*Subsets were defined as described in methods: T_N by $CD3^+CD27^+CD45RA^+$, T_{CM} by $CD3^+CD27^+CD45RA^-$, T_{EM} by $CD3^+CD27^-CD45RA^-$ and Treg by $CD3^+CD4^+CD25^+Foxp3^+$

Supplementary table 4 Intestinal lymphocyte populations at follow-up endoscopy from UC patients with endoscopic inactive UC (MAYO=0) or endoscopic active UC (MAYO>0). Medians (interquartile range) are reported.

	Inactive UC (n=7)	Active UC (n=14)	*P*-value UC patients
FU duration in months	14 (12-23)	21 (15-39)	0.149
$CD3^+$ as % of lymphocytes	55 (46-85)	50 (40-55)	0.224
$CD3^+CD4^+$ as % of T cells	51 (39-66)	78 (74-86)	0.0001
$CD3^+CD8^+$ as % of T cells	41 (25-54)	16 (11-23)	0.0001
$CD3^+CD103^+$ as % of T cells	46 (22-58)	8 (3-16)	0.0001
T_N* as % of T cells	5 (1-10)	24 (13-34)	0.0001
T_{CM}*as % of T cells	12 (7-40)	53 (45-66)	0.006
T_{EM}* as % of T cells	62 (17-81)	11 (6-24)	0.031
Treg* as % of T cells	6 (3-11)	10 (8-15)	0.056

*Subsets were defined as described in methods: T_N by $CD3^+CD27^+CD45RA^+$, T_{CM} by $CD3^+CD27^+CD45RA^-$, T_{EM} by $CD3^+CD27^-CD45RA^-$ and Treg by $CD3^+CD4^+CD25^+Foxp3^+$

Supplementary table 5. Baseline intestinal lymphocyte populations in patients with follow-up endoscopy classified as endoscopic inactive IBD (MAYO=0/ SES-CD<4) or endoscopic active IBD (MAYO>0/ SES-CD>3). Medians (interquartile range) are reported.

	Baseline % of lymphocyte subsets from patients with inactive IBD at follow-up endoscopy (n=39)	Baseline % of lymphocyte subsets from patients with active IBD at follow-up endoscopy (n=25)	*P*-value
$CD3^+$ as % of lymphocytes	52 (43-65)	57 (48-68)	0.487
$CD3^+CD4^+$ as % of T cells	69 (48-78)	74 (59-81)	0.339
$CD3^+CD8^+$ as % of T cells	25 (19-32)	23 (18-34)	0.773
$CD3^+CD103^+$ as % of T cells	14 (9-29)	13 (7-26)	0.644
T_N* as % of T cells	15 (9-34)	18 (9-27)	0.879
T_{CM}*as % of T cells	37 (26-52)	47 (38-56)	0.211
T_{EM}* as % of T cells	24 (11-47)	25 (12-32	0.784
Treg as % of T cells	10 (8-17)	12 (9-17)	0.552

Supplementary table 6. Baseline intestinal lymphocyte populations in CD patients with follow-up endoscopy classified as endoscopic inactive CD (SES-CD<4) or endoscopic active CD (SES-CD>3). Medians (interquartile range) are reported.

	Baseline % of lymphocyte subsets from patients with inactive CD at follow-up endoscopy (n=32)	Baseline % of lymphocyte subsets from patients with active CD at follow-up endoscopy (n=11)	*P*-value
$CD3^+$ as % of lymphocytes	52 (43-64)	57 (49-68)	0.371
$CD3^+CD4^+$ as % of T cells	67 (49-78)	65 (50-78)	0.880
$CD3^+CD8^+$ as % of T cells	25 (21-32)	29 (21-37)	0.315
$CD3^+CD103^+$ as % of T cells	19 (9-28)	15 (12-39)	0.573
T_N* as % of T cells	14 (8-36)	26 (15-30)	0.652
T_{CM}*as % of T cells	37 (26-50)	44 (32-50)	0.535
T_{EM}* as % of T cells	29 (11-53)	27 (20-53)	0.888
Treg as % of T cells	11 (8-17)	14 (11-18)	0.255

Supplementary table 7. Baseline intestinal lymphocyte populations in UC patients with follow-up endoscopy classified as endoscopic inactive UC (MAYO=0) or endoscopic active UC (MAYO>0). Medians (interquartile range) are reported.

	Baseline % of lymphocyte subsets from patients with inactive UC at follow-up endoscopy (n=7)	Baseline % of lymphocyte subsets from patients with active UC at follow-up endoscopy (n=14)	*P*-value
$CD3^+$ as % of lymphocytes	58 (44-70)	55 (48-65)	1.000
$CD3^+CD4^+$ as % of T cells	72 (36-87)	78 (62-83)	0.535
$CD3^+CD8^+$ as % of T cells	19 (12-48)	21 (13-29)	0.971
$CD3^+CD103^+$ as % of T cells	12 (5-30)	11 (6-17)	0.913
T_N* as % of T cells	22 (11-34)	14 (8-24)	0.393
T_{CM}*as % of T cells	33 (26-56)	53 (45-60)	0.211
T_{EM}* as % of T cells	17 (4-25)	16 (10-30)	0.643
Treg as % of T cells	9 (6-16)	10 (8-15)	0.659

5a

Integrin alphaEbeta7 in IBD: friend or foe?

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Dear editor:

We read with great interest the article of Tew et al. on the association between increased gene expression of integrin αE and granzyme A (GZMA) and clinical remission to etrolizumab treatment in patients with ulcerative colitis (UC) [1]. This report follows the recent publication of the phase two trial on etrolizumab, describing its moderate potential as induction therapy for UC [2]. Etrolizumab is a humanized monoclonal IgG1 antibody that targets integrin subunit β7 and therefore both αEβ7 and α4β7. It does not target GZMA expression. Baseline characteristics of the study by Tew et al. showed statistically significant lower endoscopy, and histology score in patients with αE^{high} gene expression compared with patients with αE^{low} gene expression. Moreover, a significant histological improvement after etrolizumab was only found in the αE^{low} gene expression group and not in the αE^{high} gene expression group. The clinical relevance of these observations is denoted as unclear, but αE^{high} expression might in fact reflect an intrinsic protective immunological mechanism in these patients. This opposes the emerging pathogenic role attributed to αEβ7 in UC by Tew et al, based on, inter alia, an unpublished paper from 2014. The role of αEβ7 in physiological conditions as well as in inflammatory bowel disease (IBD) is less distinct than that of the gut homing α4β7. In murine colitis anti-β7 has shown contradictory results [3,4]. We recently showed that newly diagnosed, untreated IBD patients had statistically significant lower percentages of αE^+ T cells in their actively inflamed intestinal biopsy specimen (Crohn's disease: 15% and UC: 11%) compared to healthy controls (30%, *P*=0.02), challenging the pathogenic role in IBD [5]. Furthermore, in contrast to the present study of Tew et al, which showed a higher gene expression of αE in anti-TNF-naïve clinical remitters, Arijs et al found no increased gene expression of αE nor β7 in IBD patients when compared to healthy controls or Infliximab responders [6]. The gene expression of the principal ligand of αEβ7, E-cadherin, was decreased in active colonic IBD when compared with healthy controls, and the use of this pathway as treatment target was seriously challenged in this paper. E-cadherin was also down regulated in active UC in the phase two etrolizumab trial, where E-cadherin expression even increased in clinical remission after treatment [2].

The authors describe increased GZMA gene expression in $CD4^+\alpha E^+$ mucosal T cells compared to $CD4^+\alpha E^-$ cells, with strikingly only 2.86% of all $CD4^+$ cells being $\alpha E^+\beta 7^+$. On the other hand, in $CD8^+\alpha E^+$ T cells, with 48.9% of all CD8+ cells expressing αEβ7, no difference is found for GZMA expression. Recently, a regulatory $CD8^+\alpha E^+$ T cell subset was found that could inhibit $CD4^+$ proliferation *in vitro* and attenuated adoptively transferred ileitis in mice [7]. In humans, alloantigen induced $CD8^+\alpha E^+$ T cells also showed functional regulatory characteristics [8]. Amongst others, etrolizumab might also affect this subset, which could explain why higher doses led to worse results with respect to clinical remission (41% in 100 mg group vs. 18% in 300 mg+LD group of GZMA high patients).

In conclusion we believe there is currently insufficient evidence to argument that αEβ7 is a "foe" in IBD. One should keep in mind that beneficial effects of etrolizumab may also rely on its effect on the α4β7 integrin. We greatly support the effort of the authors to define biomarkers/ subgroups in IBD to reach personalized medicine. However, first consistent therapeutic benefit from etrolizumab treatment in UC patients' needs to be proven in the current phase three trial.

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5b

The complexity of alpha E beta 7 blockade in Inflammatory Bowel Diseases

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ABSTRACT

Monoclonal antibodies targeting integrins are emerging as new treatment option in Inflammatory Bowel Diseases. Integrins are molecules involved in cell adhesion and signalling. After the successful introduction of anti-α4β7, currently anti-β7 is under evaluation in a phase three trial. Anti-β7 blocks both α4β7/MAdCAM-1 and αEβ7/E-cadherin interaction, targeting both the homing to and the retention in the gut of potential pathological T cells. Since the physiological and potential pathological role of immune cells expressing αEβ7 is less distinct than of those expressing α4β7. An overview of the current state of knowledge on αEβ7 in mice and human in both health and Inflammatory Bowel Diseases is presented here, also addressing the potential consequences of anti-β7 treatment.

KEYWORDS

Integrin, anti-β7, inflammatory bowel diseases, CD103, immunity

INTRODUCTION

Anti-integrin therapy targeting T-cell trafficking to the gut is evolving for patients with Inflammatory Bowel Diseases (IBD) [1]. Integrins are transmembrane molecules that mediate cell-to-cell and cell-to-extracellular-matrix interactions. They are obligate heterodimers composed of an α and β subunit, each penetrating the plasma membrane. Subunit β7 can form a heterodimeric integrin with α4 (α4β7), which can bind to Mucosal Addressin Cell Adhesion Moleclule-1 (MAdCAM-1) expressed on gut endothelial cells (figure 1). α4β7 can also bind vascular cell adhesion molecule-1 and the extracellular matrix protein fibronectin [2]. Subunit β7 can also form a heterodimeric integrin with αE (CD103) to form αEβ7. In turn, αE can only form a heterodimeric integrin with β7. For clarity, here we refer to CD103 expression when a monoclonal antibody (mAb) recognizes the αE subunit on the cell surface (by flow cytometric or immunohistochemical analysis). Since the expression of integrins on cell surface is constrained to the amount of α subunits inside the cell (as there is an excess of β subunits in the cell), and free subunits are not found on the cell surface, CD103 expression represents αEβ7 expression [3]. The only known ligand for αEβ7 is E-cadherin, an adhesion molecule on epithelial cells that is not found on endothelial cells [4]. αEβ7 expression on T lymphocytes is thought to contribute to their retention within the gut, whereas α4β7 is proposed as the major gut homing receptor on circulating lymphocytes. Anti-α4β7 blocks the gut specific α4β7/MAdCAM-1 interaction and it has been approved for the treatment of moderate to severe ulcerative colitis (UC) and Crohn's disease (CD). The mechanism of action of anti-β7 is aiming to block the binding of α4β7 and αEβ7 to MAdCAM-1 and E-cadherin respectively, rather than depleting the β7 expressing cells. Anti-β7 is mechanistically expected to have a broader, and potentially stronger, effect than anti-α4β7, given the dual approach mechanism of action [5]. By intervening with the α4β7/MAdCAM-1 interaction the anti-β7 should block the homing of $\alpha4\beta7^+$ lymphocytes to the gut, while blocking the αEβ7/E-cadherin interaction should disrupt the retention of $\alpha E\beta7^+$ lymphocytes inside the gut epithelium. However, since αEβ7 is also expressed on other immune cells and not exclusively in the gut (see below), anti-β7 treatment may have implications beyond T lymphocyte blockade. Here we present an overview of the current knowledge on αEβ7 expression and blockade in health and IBD.

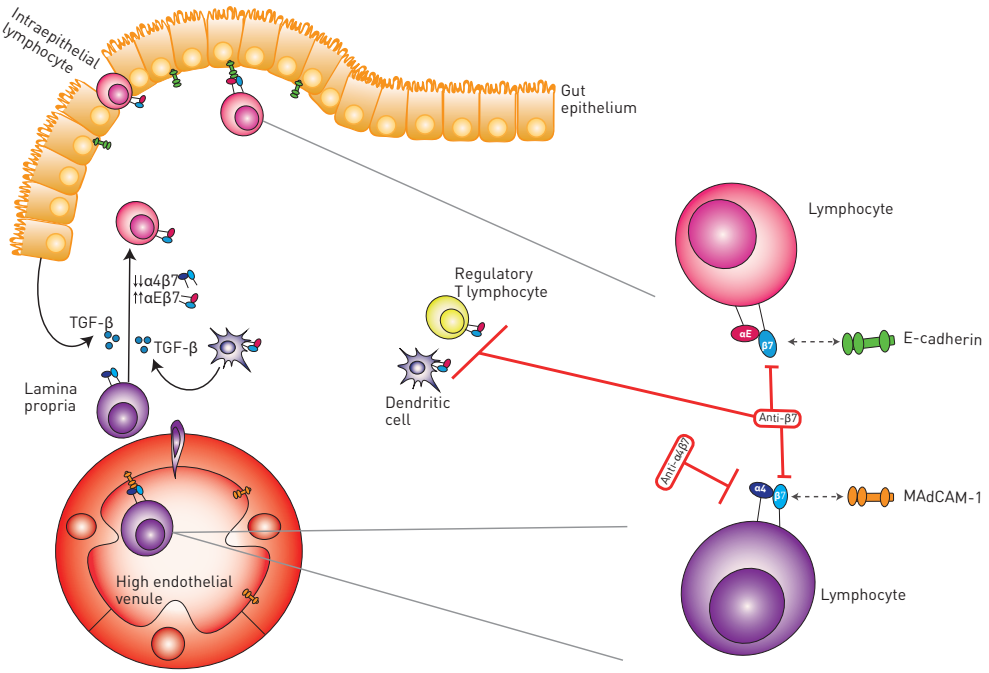

Figure 1 Integrin expression in the gut and anti-integrin treatment.

α4β7 expressing T lymphocytes enter the gut through high endothelial venules expressing MAdCAM-1. This interaction is prohibited by anti-α4β7. In a Transforming Growth Factor-β (TGF-β) dependent manner, α4β7 can be downregulated on lamina propria lymphocytes (LPL), while αEβ7 is upregulated. TGF-β is secreted by intestinal epithelial cells and αEβ7 expressing dendritic cells (DCs). Some of the αEβ7 LPLs are destined to reside in the intestinal epithelium as intraepithelial lymphocytes. They interact with E-cadherin on the epithelial cells. Anti-β7 blocks both α4β7/ MAdCAM-1 and αEβ7/E-cadherin interaction. Other immune cells like DCs and regulatory T cells can also express αEβ7 and are therefore likely to be inhibited by anti-β7.

HUMAN β7 EXPRESSING IMMUNE CELLS

Immune cells expressing α4β7 can be found in the gastrointestinal tract (oesophagus, stomach, small intestine, colon), but also in the cervix, prostate, urinary bladder, salivary gland, tonsils, thymus, lymph nodes, spleen and peripheral blood [6]. α4β7 is expressed on B and T lymphocytes (more abundantly on $CD4^+$ than on $CD8^+$ cells), natural killer cells, basophils and eosinophils, but also on innate lymphoid cells and bone marrow precursors required for the induction of tolerogenic gut-associated dendritic cells (DCs) [7-11]. The proportion of $CD4^+$ T lymphocytes in healthy human blood co-expressing CD45RO (memory phenotype) and α4β7 was shown to be

36% [12]. By blocking α4β7, one could expect a reduced recruitment of T lymphocytes into the intestine, given its high specificity for gut homing. However, it would also effect other immune subsets expressing α4β7 (as discussed in [13,14]). After anti-α4β7 treatment in IBD patients, saturation of peripheral $CD45RO^+CD4^+\alpha4\beta7^+$ T lymphocytes was 90-95%[15,16]. There were no data presented on the examination of other cell subsets expressing α4β7 or of T lymphocyte subsets in biopsy specimens [15,16]. Effects of anti-β7 treatment on T lymphocytes are discussed below. B cells were shown to accumulate in the periphery after β7 blockade (absolute numbers), but neither intestinal effects nor effects on other $\alpha4\beta7^+$ immune subsets were examined [17].

Diverse immune cell subsets can induce CD103 (and therefore αEβ7) expression on their surface. These cells can be found in the spleen, thymus, skeletal muscle, heart, liver, kidney, skin, brain, gastrointestinal tract, urogenital tract and lungs [18-25]. In the intestinal mucosa, the $CD103^+$ immune cell subsets include intraepithelial T lymphocytes (IELs, CD103 expressed on the vast majority of IELs, mainly on $CD8^+$ IELs) [24-26], lamina propria T lymphocytes (LPLs, CD103 expressed on about half of LPLs, mainly on $CD8^+$ LPLs) [24-26], subsets of DCs [27-30], intraepithelial innate lymphoid cells type 1 [31], and regulatory T lymphocytes [32,33] (see figure 1). CD103 expression has been described on colonic $CD4^+CD25^+FoxP3^+CD127^{low}CTLA\text{-}4^+$ regulatory T cells (phenotypic Tregs) [32]. In addition, a regulatory alloantigen-induced $CD8^+CD103^+$ T-cell subset has been described in humans [34]. Furthermore, intestinal epithelial cells were shown to induce suppressive $CD8^+CD103^+$ cells [33], suggestive of a regulatory $CD103^+$ subset in the intestinal mucosa [33,34]. The same research group underlined the relevance of these cells in maintaining mucosal tolerance, when they demonstrated the absence of this $CD8^+$ regulatory activity in both UC and CD [35]. Next to migratory subsets of T lymphocytes (central memory and effector memory) expressing α4β7 to grant them access to the gut, it has become clear that another, non-migratory, subset of T lymphocytes exists: tissue-resident memory T cells (T_{RM}) [36]. These T_{RM} cells reside in epithelial barrier tissues and deliver a prompt response to former encountered pathogens, independent of recruitment of migratory T cells from the blood. Therefore, T_{RM} mediate the rapid protective immunity to infectious agents, which is a hallmark of adaptive immune memory. Migratory T cells can, when necessary, replenish the T_{RM} compartment. CD103 has been described on a subset of T_{RM} cells, more predominant on $CD8^+T_{RM}$ than on $CD4^+T_{RM}$ cells (approximately 88% of $CD8^+$ LPL and 38% of $CD4^+$ LPL in one human study [25] and 80% of $CD8^+$ cells versus 15% of $CD4^+$ cells in healthy human ileum and colon in another study [37]). Around 40% of $CD103^+$ cells in the gut were shown to express T_{RM} marker CD69 [30]. CD103 (αEβ7) expression on peripheral blood T lymphocytes is virtually absent (<2%) [37]. Expression of CD103 on $CD4^+$ and $CD8^+$ T lymphocytes is mediated by the anti-inflammatory cytokine Transforming Growth Factor-β (TGF-β) [30,38,39] (figure 1). By blocking CD103, one could expect a reduced retention of T lymphocytes within the intestine, in particular the $CD8^+$ subset, but also the T_{RM} and Treg subpopulation. However the role and function of CD103 positivity of T cells, including T_{RM} and Treg, in chronic inflammation has not yet been elucidated. Effects of specific α4β7 and αEβ7 blockade on the migration and retention

of different cell subsets would therefore be an interesting topic of investigation. Furthermore, since αEβ7 is not as gut-specific as α4β7, retention of αEβ7 expressing cells in other barrier tissues such as the lung and skin would also be affected. Even though the implications of β7 blockade for other organs are still unclear, this might lead to unwanted side effects of anti-β7 treatment in IBD patients.

REGIONAL DIFFERENCES IN β7 EXPRESSION ALONG THE INTESTINE

Intestinal disease location differs greatly between different clinical phenotypes of UC and CD. Therefore, potential differences in the number of CD103 expressing immune cells in different bowel segments are relevant, and should be addressed when considering anti-β7 treatment (see figure 2). A regional difference of CD103 expressing cells was found in the gut of rhesus macaques with more $CD103^+$ cells in the small intestine (±35% $CD103^+$ cells/mm epithelium) than in the colon (±25%) [40]. This correlated well with CD3 positivity, implying a decline of IEL numbers along the macaques' intestine. The same results were found in healthy humans, with higher percentages of CD103 expressing IEL and LPL in ileum (median 84% $CD103^+$IEL and 42% $CD103^+$LPL) versus colon (median 56% $CD103^+$IEL and 22% $CD103^+$ LPL) [41]. These findings are in line with another study describing a linear decrease of the number of cells expressing CD103 from ascending colon to rectum in healthy humans, which was contrarily to what the authors expected [42]. Therefore, a direct relation between the number of $CD103^+$ cells and the disease distribution in UC (increasing severity from distal to proximal colon) seems unlikely. Moreover, β7 blockade might show less effect in patients with disease limited to the rectum (proctitis) compared with involvement of the entire colon (pancolitis).

In contrast with the decreasing presence of $CD103^+$ T lymphocytes along the intestine, a reversed presence of CD103 expressing DCs was recently reported in humans, with lower proportions of $CD103^+$ DCs in the proximal compared with distal colon [43]. These authors also showed that healthy human colonic $CD103^+$ DCs co-express β7 [43].

In healthy human ileum, α4 was expressed on 32% of IELs and 35% of LPLs whilst in left colon it was expressed on 48% of IELs and 58% of LPLs [41]. In another study, the proportion colonic T lymphocytes in healthy humans expressing α4β7 was 72% [12].

It remains to be determined how different $\beta7^+$ immune cell subsets are distributed along the intestine, but it would be important to assess their location and function profile in order to fully understand the consequences of their blockade.

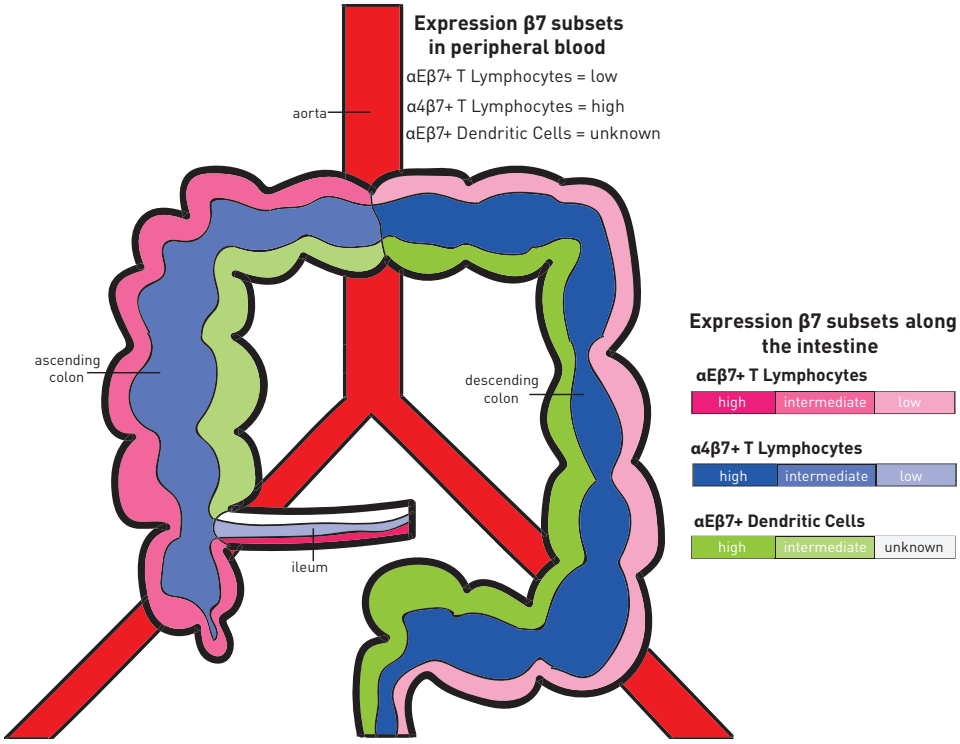

Figure 2. Healthy human expression of β7 immune subsets along the intestinal wall and in peripheral blood.

The number of T lymphocytes expressing αEβ7 is highest in ileum, with a descending gradient along the intestine showing lower numbers in de descending colon. On the other hand, frequencies of dendritic cells expressing αEβ7 are lower in the ascending colon than in the descending colon, with unknown expression in ileum. T lymphocytes expressing α4β7 are lower in ileum and higher in descending colon. $\alpha E\beta 7^{+}$ T lymphocytes are virtually absent in the peripheral blood compartment, with a more substantial presence of $\alpha 4\beta 7^{+}$ T lymphocytes.

DENDRITIC CELLS EXPRESSING CD103

Dendritic cells are strategically positioned in the intestinal mucosa to capture antigen and play a key role in initiating and modulating immunogenic and tolerogenic responses. Different subsets can be discriminated by expression of surface markers and their functional characteristics. The subset of myeloid DCs ($CD11c^{+}CD123^{-}HLADR^{+}$) co-expressing CD103 is specialized in generating regulatory T cell responses via TGF-β and retinoic acid [44,45]. In mice, specific subsets of DCs were identified, with different capacity of peripheral Treg induction [46]. Both subsets of DCs ($CD103^{+}$ and $CD103^{-}$) induced expression of the gut homing integrin

α4β7 on $CD4^+$ T cells, but only $CD103^+$ DCs were able to induce expression of C-C chemokine receptor 9 (CCR-9) on T cells [47]. CCR-9 is a homing marker for the small bowel, as it binds to C-C motif ligand 25 on epithelial cells of the small intestine. Further investigation showed only $CD103^-$ DCs to be able to induce effector T lymphocytes that secreted proinflammatory cytokines (interleukin-6 and tumour necrosis factor-α (TNF-α)), whereas $CD103^+$ DCs were responsible for inducing functionally suppressive $FoxP3^+$Tregs [44]. In mice, CD103 expression on DCs was found to be mandatory for the induction of Tregs that suppressed colitis and attenuated ileitis [47,48]. The presence of a tolerogenic $CD103^+$ DC population, able to drive Treg development, was also identified in the human gut [29]. The literature regarding effects of DCs in CD103 knockout mice is discussed below. Lack of β7 expression in the innate immune compartment resulted in accelerated T cell-mediated colitis in $\beta7^{-/-}RAG2^{-/-}$ mice, correlating with lower numbers of retinoic acid producing DCs [9]. Furthermore, lack of $\beta7^+$ DCs impaired induction of $Foxp3^+$ and IL-10 producing Tregs [9]. In a T cell-mediated model of colitis, a subset of $CD103^-$ DCs accumulating in the inflamed intestine, were shown to express E-cadherin and to produce colitogenic cytokines upon activation [49]. After Fms-related tyrosine kinase 3 ligand administration this DC subset decreased in the lamina propria, whilst $CD103^+$ DCs increased, resulting in the induction of $CD4^+CD25^+FoxP3^+$ Tregs and attenuation of murine ileitis [48]. We hypothesize that the decrease of proinflammatory $CD103^-$E-$cadherin^+$ DCs might be mediated by regulatory interaction of CD103 (DCs and Tregs) with E-cadherin resulting in immune suppression.

Of note, Foxp3 is known to be a specific marker for functionally suppressive $CD4^+$ Tregs in mice [50]. The role of this marker for Tregs in human remains controversial, as activation of non-Tregs can lead to transient Foxp3 expression without inducing a suppressive function [51]. This does not concern the interpretation of the data presented here, as it mostly regards Tregs in mice, but caution should be taken when interpreting human data on this subject.

A dramatic loss of $CD103^+$ intestinal DCs was observed during experimental colitis ($CD4^+CD62L^+$ transfer model and chronic Dextran sulphate sodium-colitis) in the lamina propria and mesenteric lymph nodes (MLN) compared with healthy mice [52]. Recently, this was reaffirmed in Dextran sulphate sodium-colitis mice, with decreased frequencies of $CD103^+$ DC subsets associated with a reduced ability to promote Treg generation [53]. $CD103^+$ DCs with similar phenotype and functions as in mice were also found in human MLN [30,44]. Reduced numbers of $CD103^+$ DCs were also described in the colonic biopsy specimen of patients with active UC compared with healthy controls [54]. Similarly, statistically significant lower numbers of $CD103^+$ DCs were reported in inflamed compared with non-inflamed gut mucosa of CD patients [55]. The $CD103^+$ DC population increased statistically significant after anti-TNF-α treatment [55].

Taken together, these findings support a role for $CD103^+$ DCs in the intestinal maintenance of immune homeostasis, as they are involved in Tregs induction in the gut. Therefore, blocking β7 would not exclusively block $\alpha E\beta7^+$ on T lymphocytes, but might also affect the $CD103^+$ subset of

DCs and β7^{+} innate immune cells, thereby exercising a potentially negative influence on long-term immune homeostasis.

The process of Tregs induction by CD103^{+} DCs is dependent on TGF-β and potentiated by vitamin A metabolite retinoic acid. On the other hand, TGF-β can also induce formation of Th-17 cells in the presence of the pro-inflammatory cytokine IL-6 [56] (see figure 3). Furthermore, as described earlier, TGF-β induces CD103 expression on both CD4^{+} and CD8^{+} T lymphocytes [30,38,39]. TGF-β signalling is regulated through the SMAD-pathway. Overexpression of SMAD7 in transgenic mice completely impaired the TGF-β mediated up-regulation of CD103 expression on CD4^{+} T cells and reduced the number of intestinal IEL in these mice by 46%, compared with their wild-type littermates [57]. To our knowledge, this has not yet been studied in humans. However, overexpression of SMAD7 was reported in the inflamed intestine of IBD patients, concomitantly to abnormally decreased TGF-β1 activity [58]. An oral antisense therapy inhibiting SMAD7 was remarkably effective in inducing remission and clinical response in a phase two trial in CD patients [59]. It is tempting to speculate that the inhibition of SMAD7 could ensure an increased functional TGF-β activity and might result in up-regulation of CD103 expression on T lymphocytes [57,60].

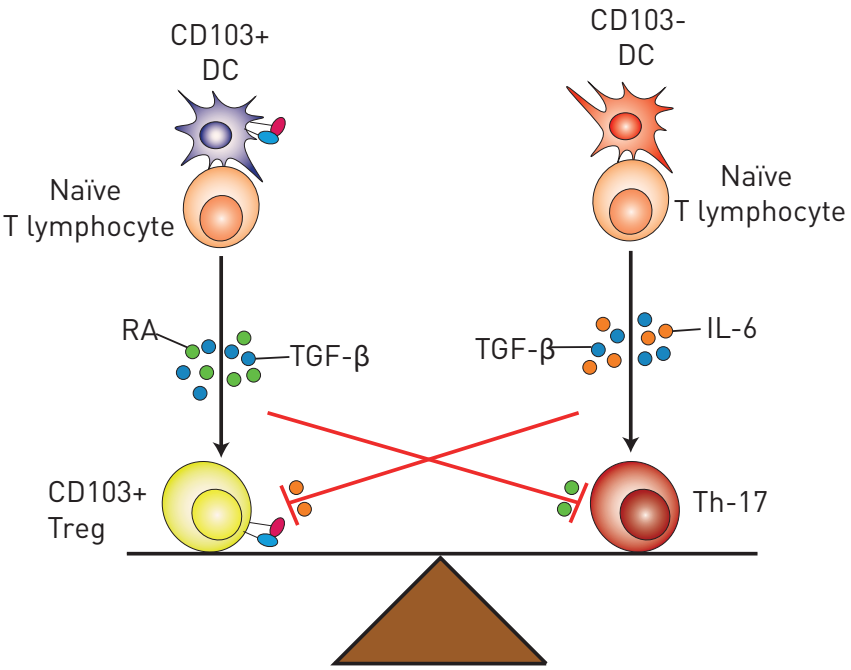

Figure 3. Transforming Growth Factor- β (TGF-β) dependent immune balance between pro- and anti-inflammatory responses in the gut.

$CD4^+CD25^+FoxP3^+$ regulatory T lymphocytes (Tregs) can emerge when $CD103^+$ dendritic cells (DCs) present antigen to naïve T lymphocytes (T_N). This process is dependent on the presence of TGF-β and retinoic acid (RA). On the other hand, Th-17 lymphocytes are formed when $CD103^-$ DCs present antigen to T_N in the presence of TGF-β and the proinflammatory cytokine interleukin-6 (IL-6). Additionally, IL-6 reduces Treg induction and RA reduces Th-17 lymphocyte induction.

CD103 AND E-CADHERIN EXPRESSION IN IBD PATIENTS

A detailed overview of different papers studying CD103 expression in IBD patients can be found in table 1. One study examined CD103 expression on IELs and LPLs in surgical colonic resection specimen of patients with CD (disease activity and location unclear), UC (both active and inactive disease, disease location unclear) and controls (colectomy due to colorectal carcinoma) [61]. No difference in CD103 expression on IELs was observed between IBD patients and controls. Higher numbers of $CD103^+$ LPLs were found in CD patients compared with UC patients and controls. In this study no difference regarding E-cadherin expression was found between patients and controls. Another study was performed on CD103 expression on IEL and LPL in the ileum and colon of CD patients (active and inactive disease taken together)

and healthy controls [41]. In contrast with the aforementioned study, no difference was found for CD103 expression on LPL between CD and controls. Furthermore, decreased expression of CD103 was found on IEL in the ileum of CD patients compared to controls. The only cell subset with a statistically significant overexpression of CD103 was the $CD3^+CD25^+$ LPL of the left hemicolon, which also include Tregs. The same research group performed a pilot study in patients with CD, UC (partly under treatment with mesalazine and/or corticosteroids) and healthy controls [62]. IL-2 expanded T cells were generated from inflamed colonic biopsy specimens. After expansion, increased CD103 expression was observed on $CD3^+$ and $CD8^+$ cells, but not on $CD4^+$ cells, in CD patients compared to controls. In UC patients this was only demonstrated on the $CD8^+$ subset and not $CD4^+$ or overall $CD3^+$ cells.

We previously described lower percentages of $CD103^+$ T lymphocytes in actively inflamed intestinal biopsy specimens of newly diagnosed, untreated IBD patients (CD and UC), compared with biopsy specimens of healthy controls (P=0.02) [63]. Recently, immunohistochemical analysis of CD103 expression in gut samples of unknown location demonstrated higher percentage of $CD4^+CD103^+$ cells in treated IBD patients compared to controls [30]. However, the percentage of $CD103^+CD8^+$ cells did not differ between patients and controls. Altogether, there is no strong evidence for increased CD103 expression on T cells in the gut of IBD patients. At the level of gene expression of cell adhesion molecules, no statistically significant difference was found for gene expression of αE and β7 in the (inflamed) mucosa between active IBD patients and healthy controls or post-Infliximab treatment [64]. Gene expression of E-cadherin in inflamed colonic samples of IBD was statistically significant decreased compared to healthy controls. The authors seriously challenged the use of this pathway as treatment target, as they observed no upregulation of αE or β7 in the inflamed IBD mucosa next to down-regulation of its principal ligand E-cadherin. On the other hand, support for the use of anti-α4β7 in patients with IBD was found in this study, as α4 and MAdCAM-1 gene expression was significantly increased in active inflammation and both decreased in Infliximab responders after therapy.

Results on intestinal E-cadherin expression in IBD are also inconsistent, but most studies advocate a decreased expression. As mentioned, E-cadherin gene-expression was downregulated in active colonic IBD when compared with healthy controls [64]. In line with this, immunohistochemical staining of colonic specimen of IBD patients showed decreased staining of E-cadherin at the mucosal edges of ulcerations in all active UC patients and in half of active CD patients. Non-ulcerated and non-inflamed mucosa showed normal E-cadherin expression [65]. E-cadherin was downregulated in another study performed in UC patients [66]. On the other hand, E-cadherin was upregulated (immunohistochemistry and mRNA) at sites adjacent to ulcerations, with absence inside the ulceration of ileal and colonic biopsy specimen of IBD patients with active disease [67]. E-cadherin expression is likely to be adjusted to local CD103 expression. It is conceivable that decreased E-cadherin expression in gut mucosa of IBD patients might diminish the effect of anti-β7 treatment on retention of $CD103^+$ T lymphocytes within the epithelium.

Overall, the knowledge on CD103 and E-cadherin expression in IBD patients is most likely blurred by inhomogeneous patient cohorts including both active and inactive disease and under various types of anti-inflammatory treatment. Examining CD103 and E-cadherin together might provide more clarity on their expression and interaction. Furthermore, studies have only addressed CD103 expression on the total T cell population, while subpopulations may differ in expression levels and may also be affected differently by anti- β7 treatment. So far there is no convincing evidence of increased CD103 or β7-expression on T cell (subsets) in the inflamed gut mucosa of IBD patients.

Table 1. CD103 expression in the intestinal mucosa of IBD patients.

Method	Patients	Disease specifications	Results on CD103	Other results
IEL and LPL obtained from biopsy specimen by enzymatic extraction, analysed by FACS [41]	CD (n=18), HC (n=20)	• Active and inactive disease on one heap: CDi 4 inflamed & 8 non-inflamed; CDc 14 inflamed & 12 non-inflamed • Patients on mesalazine, steroids, azathioprine or no treatment • Disease duration unknown	• **IEL: ↓CD103+ in CDi (53%) vs HC (84%) p<0.05**, colon NS (30% vs 58%) • LPL: overall CD103$^+$ CD (39% ileum, 26% colon) =HC (42% ileum, 22% colon). **↑CD103+ in CD3+CD25+ of left colon CD (12%) vs HC (5%) p<0.05**	-
Immunohistochemical staining and quantification of surgical colonic specimen on CD103 positivity [61]	CD (n=12), UC (n=12), HC (n=5)	• UC: active and inactive disease together: 9 active, 3 inactive CD: disease activity not described • All UC and 6/12 CD patients on steroids • Disease duration unknown	• IEL: HC=UC=CD (2.5-3.5% of 1000 epithelial cells) • **LPL: ↑CD103 in CD (12% of 500 mononuclear cells) versus UC (4%) p<0.0001 and HC (4%) *P*=0.0006**	E-cadherin HC=UC=CD
FACS analysis on expanded T cells of 43 cell lines generated from colonic biopsy specimen by IL-2 expansion [62]	CD (n=4), UC (n=5), HC (n=6)	• Active disease, mesalazine and steroids/untreated taken together • Disease duration unknown	• **↑CD3+CD103+ in CD (12%) versus HC (3%), *P*=<0.05.** UC (9%), NS. • **↑CD8+CD103+ CD (42%) and UC (26%) versus HC (6%), *P*=<0.05.** CD4$^+$CD103$^+$ CD (6%) =UC (1%) = HC (10%)	**↓ α4β7 in CD (36%) versus controls (93%).** α4β7 in UC(78%)=HC Pilot study

Table 1. continued

Method	Patients	Disease specifications	Results on CD103	Other results
FACS analysis of biopsy specimen with CD103 staining of CD3 cells [63]	CD (n=51), UC (n=14), HC (n=14)	• Active disease • Untreated patients • Newly diagnosed patients	• **↓ CD103+CD3+ in CD (15%) and UC (11%) versus HC (30%), *P*=0.02**	-
Immunohistochemical analysis of gut samples with CD103, CD4 and CD8 staining [30]	CD (n=19), UC (n=12), HC (n=14)	• Active disease, partly with immunosuppressants and biologicals • Disease duration unknown	• **↑ CD4+CD103+ in CD (14%) and UC (10%) versus HC (5%), *P*<0.01 and *P* <0.05** • $CD8^+CD103^+$ CD=UC=HC (35-45%) and $Foxp3^+CD103^+$ CD=UC=HC (4-7%)	
Mucosal gene expression in biopsy specimen. Biopsies taken in the week before 1st IFX infusion and 4-6weeks after 1st infusion [64]	UC (n=24), CDi (n=18), CDc (n=19), HC (n=12)	• IBD refractory to steroids and/or immunosuppression starting IFX treatment • Median disease duration varying from 6.4-22.3 years	• UC=CDc=CDi=HC for both CD103 (ITGAE, fold change 1.05, *P*=0.625) and beta7 (ITGB7, fold change 1.15, *P*=0.187), no change after IFX treatment	E-cadherin down regulated (CDH1, fold change 0.41, *P*=0.002) **↑α4 and ↑MAdCAM-1 in active inflammation fold change respectively 3.17, *P*=0.00001, and 1.63, *P*=0.001), both decreased in IFX responders (fold change respectively 0.57 and 0.77)**

Bold = statistical significant difference, IBD= inflammatory bowel diseases, CD= Crohn's Disease, UC= Ulcerative Colitis, HC= healthy controls, CDi= ileal CD, CDc= colonic CD, IFX= infliximab, FACS= Fluorescence-activated cell sorting

LESSONS FROM αEβ7 IN MOUSE MODELS

Most of the current knowledge on αEβ7 blockade is derived from animal models. Anti-β7 treatment was shown to reduce (but not heal) colitic lesions in a $CD45RB^{high}CD4^+$ T cell transfer model of murine colitis [68]. In a study on anti-β7 treatment in SCID mouse after T cell transfer, mice were sacrificed 1.5 hours after anti-β7 administration [5]. In this model, lower numbers of lymphocytes were found in the inflamed colon of treated mice and there was no apparent effect on the spleen. However, the observational period was too short to determine long-term effects. Contrarily, another study showed that the transfer of naïve T cells from β7 knockout mice into SCID mice only delayed the development of murine colitis (symptoms started after 20 weeks instead of within 3-4 weeks) and did not alter the course of disease [69]. β7 expression was not even a strict requirement for the localization of T lymphocytes to the mouse intestine (β7 knockout mice) and it was suggested that alternate pathways might compensate a lack of β7. Another study on β7 knockout mice demonstrated less pronounced colitis compared to wild-type mice, coinciding with decreased numbers of mucosal proinflammatory monocytes [70]. Progenitors of these monocytes were shown to express β7, but expression of α4β7 and/or αEβ7 was not studied individually. Anti-β7 treatment and β7 knockout models affect α4β7 and αEβ7 expressing cells simultaneously. This impairs differentiation between the specific effects of anti-β7 treatment on homing (α4β7) or retention (αEβ7) of immune cells.

Anti-CD103 treatment was able to prevent the development of colitis and ameliorated existing colitis in a TNP-OVA-immunized IL-2 knock-out mouse model [71]. When mice were sacrificed 7 days after the first anti-CD103 injection a reduction of $CD4^+$ LPLs was seen, but, surprisingly, there was little effect on $CD8^+$ T lymphocytes. Moreover, the treatment led to inflammation of the spleen. Another study failed to confirm any role of CD103 expressing T lymphocytes in the development of colitis and showed that accumulation of $CD4^+$ T cells in the intestine did not require CD103 expressing T lymphocytes [47]. Colitis developed normally in the absence of CD103 expressing T lymphocytes in a T cell transfer mouse model and regulatory $CD103^-$ T cells were still able to suppress colitis [47].

Overall, there is a broad range of effects in mice treated with anti-β7 or anti-CD103 and retention of T lymphocytes in the gut seemed to be compensated by influx of migratory T cells, in the absence of CD103 and β7.

With respect to DC subsets, similar composition was found in $Rag2^{-/-}$ mice and $CD103^{-/-}$ $Rag2^{-/-}$ mice, but the function of these DCs was not analysed. In another study, $CD103^{-/-}$ mice with a helminth infection displayed comparable frequencies of DCs with wild type mice [72]. Unfortunately, the ability of these DCs to generate retinoic acid, Tregs and α4β7/CCR9 expressing T lymphocytes was not analysed. On the other hand, in mice with induced diabetes, anti-CD103 immunotoxin (with a depleting effect on $CD103^+$ cells) resulted in a dramatic loss of DCs and Tregs in the mesenteric lymph nodes [73]. Therefore, even though the number of DCs present after CD103 knockout induction in mice, does not seem to differ, it is conceivable that

these DCs might have a more proinflammatory function. Another explanation could be, that DCs need β7 to exert their function of inducing suppressive Tregs or to block the function of $CD103^-$E-$cadherin^+$ DC subsets [11].

A regulatory $CD8^+CD103^+$ T cell subset that could inhibit $CD4^+$ proliferation in vitro and attenuate adoptively transferred ileitis has also been described in mice [74]. This was confirmed in another study, in which is shown that only the $CD8^+CD103^+$, not the $CD8^+CD103^-$, T lymphocyte subset could suppress T-cell mediated colitis in $Rag2^{-/-}$ mice [75]. Remarkably, numbers of regulatory $CD8^+CD103^+$ blood T lymphocytes increased gradually during a year of anti-α4 treatment in patients with multiple sclerosis, more significantly in patients with clinical response [76]. These data show evidence of a regulatory $CD8^+CD103^+$ T cell subset. The effects of anti-β7 treatment on these subsets have not yet been evaluated.

ANTI-β7 TREATMENT IN IBD PATIENTS

The rationale behind anti-β7 treatment is based on mice studies and some of the studies described in table 1, under the assumption of an upregulated CD103 expression in the inflamed intestine of IBD patients [41,61,62]. The phase one trial of anti-β7 in IBD included 48 patients with UC in order to assess safety and tolerability [77]. Overall, it was well tolerated with headache as most common adverse event. Clinical response was observed in 12/18 anti-β7 treated patients (4/5 in placebo group), with clinical remission in 3/18 anti-β7 treated patients (1/5 placebo group). The phase two trial tested anti-β7 in 119 UC patients in two dosages: 100mg and 300mg compared with placebo [17]. Administration of anti-β7 led to maximal occupation of β7 receptors on circulating T lymphocytes ($CD4^+$ and $CD8^+$) with a subsequent increase of $CD4^+\beta7^+$ numbers in the peripheral blood in line with the hypothesis that anti-β7 interferes with recruitment of effector T cells to the intestine. Peripheral $CD8^+\beta7^+$ frequencies remain the same. In the inflamed colonic mucosa the β7 receptors on $CD8^+$ T lymphocytes were maximally occupied (both αEβ7 and α4β7). However, frequencies of these lymphocytes remained comparable with placebo treated patients. Unfortunately, $CD4^+\beta7^+$ T lymphocytes were not analysed in the colonic biopsy specimens. Intestinal biopsy mRNA levels of αE, β1 and β7 were unchanged. Therefore the mechanism of action of anti-β7 seems more blocking than depleting. Clinical remission at week 10 was observed in 8/39 patients with 100mg, 4/39 patients with 300mg and 0/41 patients with placebo. A simultaneous endoscopic and rectal bleeding subscore of 0 at week 10 was found in 4/39 patients with 100mg, 3/39 with 300mg and 0/41 with placebo. An indirect comparison, posed in the discussion of this phase two trial, of anti-β7 with the phase two trial of anti-α4β7 [15] indicated a higher placebo-corrected clinical remission in patients treated with anti-β7 (21% compared with 18.5%). However, this was an unequal comparison, since the endpoint after 6 weeks of treatment in the anti-α4β7 trial was compared to the endpoint after 10 weeks in the anti-β7 trial. The placebo-corrected clinical remission in the 100mg anti-β7

group after 6 weeks of treatment was much lower (5%) than in the anti-α4β7 trial (18,5%). The same trend was seen when making an indirect comparison for endoscopic remission: at week 6 the placebo-corrected endoscopic remission was 20% in the anti-α4β7 trial as opposed to 5% in the anti-β7 trial. Of note, patient cohorts studied were different regarding disease severity, refractoriness to previous medication and both studies allowed co-medication during the study period. Obviously, final comparison can only be made in a head-to-head trial studying both mAbs.

In a recent post-hoc study on the potency of biomarkers in IBD patients treated with anti-β7, gene expression of integrin alpha E (ITGAE) and granzyme A were studied as potential predictive markers of response [78]. Baseline characteristics showed lower endoscopy- and histology scores in patients with $ITGAE^{high}$ compared with $ITGAE^{low}$ patients. The clinical relevance of this observation was denoted as unclear, but we wonder if the $ITGAE^{high}$ expression could in fact reflect a predominant anti-inflammatory pathway in these patients? Histological improvement was only found in the $ITGAE^{low}$ group and not in the $ITGAE^{high}$ group.

Considering the earlier mentioned gradual decline of $CD103^{+}$ T lymphocytes along the intestine, it might be possible that there are other identifiable subgroups of patients who might benefit from anti-β7 treatment (e.g. ileal disease when compared with distal colonic disease). On the other hand, in view of αEβ7 expression on specific regulatory immune cell subsets, comprehensive evaluation of these subsets might also uncover markers to stratify patients for personalised medicine. It would be of interest to determine the actual integrin expression on the surface of all different subsets of immune cells in the gut by, for example, FACS analysis before and after anti-β7 treatment.

CONCLUSION

Intervening in the gut specific infiltration of T lymphocytes ($\alpha4\beta7^+$) is a very effective approach to directly address the site of inflammation in IBD. On the other hand, the effect of blocking retention of T lymphocytes ($\alpha E\beta7^+$), which already populate the inflamed intestinal mucosa, is less clear. In addition, the effect of anti-β7 treatment on the diverse immune subsets expressing αEβ7 in the peripheral blood as well as the different epithelial compartments has not yet received sufficient attention.

We believe that, given the current knowledge, there is no evidence for an exclusively pathogenic role of αEβ7 positive intestinal immune cells in IBD, as there is no consistent evidence for increased αEβ7 expression on lymphocytes in patients. Moreover, αEβ7 integrin blockade might interfere with the action of different regulatory immune cell subsets. Conflicting results have been reported in different mouse models regarding treatment with anti-CD103 and anti-β7 mAb, therefore the results of the phase three anti-β7 trial are eagerly awaited. In the meantime, more comprehensive studies on αEβ7 expression in IBD are needed to elucidate its true nature and eventually define specific subgroups of patients that might benefit from anti-β7 treatment. Furthermore, it is essential to take into account the possible effects of αEβ7 blockade on long-term immune homeostasis, as well as side effects on other organs and the response to intestinal infections.

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6

The value of serum antibodies in differentiating inflammatory bowel disease, predicting disease activity and disease course in the newly diagnosed patient

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ABSTRACT

Background

Data on serum antibodies in untreated adult inflammatory bowel disease (IBD) patients at diagnosis are scarcely available, and results on the stability of antibody presence over time are inconsistent. Our aim was to investigate antibodies in newly diagnosed, untreated IBD patients in relation to disease phenotype and course. Furthermore, we analyzed antibody presence over time.

Methods

Baseline anti-*Saccharomyces cerevisiae* antibodies (ASCA), anti-chitobioside carbohydrate antibodies (ACCA), anti-laminaribioside carbohydrate antibodies (ALCA) and anti-mannobioside carbohydrate antibodies (AMCA) were measured with enzyme-linked immunosorbent assays and perinuclear anti-neutrophilic cytoplasmic antibodies (pANCA) was measured by indirect immunofluorescence in serum of 120 untreated IBD patients at diagnosis and 19 healthy controls. Antibodies were related to disease outcomes. Serial measurements were available in 71 patients.

Results

The combination of pANCA and ASCA enabled good discrimination between UC and CD (P=0.004). Antibody presence was relatively stable over time, even though there were significant changes in concentrations. There was a trend towards larger fluctuations in concentration with immunosuppressive medication. Baseline pANCA in UC patients correlated with calprotectin values (rho=0.545, P=0.019) and change in pANCA status over time was associated with disease activity at that moment. No associations were found with antibodies at diagnosis and disease outcomes.

Conclusion

Antibody profiles at diagnosis support the distinction between CD and UC. Anti-glycan antibodies are reasonably stable over time, but may fluctuate under the influence over immunosuppressive treatment which may explain the inconsistency in findings hitherto. The appearance or disappearance of pANCA antibodies during follow-up correlated with disease activity in UC and may be used in disease monitoring.

INTRODUCTION

Inflammatory bowel diseases (IBD) covers a heterogeneous group of chronic, relapsing-remitting diseases of the gastrointestinal tract, classified as Crohn's disease (CD), ulcerative colitis (UC) and IBD type unclassified (IBDU). CD and UC diagnosis are based on a combination of clinical, endoscopic, histological and radiological criteria [1,2]. IBDU is diagnosed in 10-15% of patients, in whom there is diagnostic uncertainty and in the absence of resection material of ileum or colon, which may influence clinical decision making [3,4]. In IBD patients, it is difficult to predict disease course or response to treatment at initial diagnosis and during follow-up. Early identification of IBD patients at risk for a severe disabling disease course could allocate those in need for early top-down treatment. Easily accessible, objective, markers for disease activity or that predict future disease course would substantially contribute to early patient stratification in IBD.

Serological antibodies have been investigated extensively in IBD patients for many years [5,6]. Simultaneous measurement of anti-*Saccharomyces cerevisiae* antibodies (ASCA) and perinuclear anti-neutrophilic cytoplasmic antibodies (pANCA) was shown to carry high specificity, but low sensitivity, in differentiating CD ($ASCA^+$, $pANCA^-$) from UC ($ASCA^-$, $pANCA^+$) [7]. These antibodies might be helpful in further classification of IBDU patients, but their routine use in IBD patients and their role as biomarker for disease activity is still not clear [8,9]. Recently, it was found that patients with higher number and titers of antimicrobial markers before diagnosis were at highest risk of presenting with complicated disease [10]. New anti-glycan antibodies like anti-chitobioside carbohydrate antibodies (ACCA), anti-laminaribioside carbohydrate antibodies (ALCA) and anti-mannobioside carbohydrate antibodies (AMCA) have been identified in IBD patients [11-13]. Anti-glycan antibodies were shown to be predominantly present in CD patients with ileal disease and in patients with a complicated disease phenotype (stricturing and/or penetrating disease behavior). However, in previous papers on adult IBD patients, these antibodies were mainly analyzed in cross-sectional, single time point studies, when complications had already occurred and treatment was already commenced [12-17]. Results on baseline concentrations at primary diagnosis are scarcely available and mainly derived from pediatric cohorts [18,19]. Therefore the value of serological autoantibodies in IBD patients can merely be considered to reflect, rather than predict, complicated disease phenotype [20]. Only a few studies have evaluated the evolution of antibodies over time (longitudinally), but no definite statement on their stability of antibody presence over time can be made, as serum samples were not assessed at diagnosis [21]. Although some papers claim stability over time, mixed associations with disease duration, disease activity and drug therapy have been found, contradicting antibody stability [20].

In other immune mediated diseases, serological antibody evaluation is used during disease follow-up [22-27]. In IBD patients no definite conclusions can be drawn on the use of serological

antibodies, neither as marker for disease activity during follow-up, nor as predictive biomarker at diagnosis for disease progression.

We aimed to investigate a panel of candidate serological antibodies in newly diagnosed, untreated, adult IBD patients at diagnosis and during the course of disease, by assessing their presence and serum concentration over time in relation to disease activity, severity and response to therapy.

MATERIAL AND METHODS

Study population

IBD patients were prospectively enrolled and longitudinally followed-up at the outpatient clinic of the Department of Gastroenterology and Hepatology at Rijnstate Hospital Arnhem, a secondary care hospital in the Netherlands. Patients were recruited prior to the initial diagnostic ileocolonoscopy. After standardized work-up with clinical, endoscopic, histopathological and, when applicable, radiological evaluation, all patients met the generally accepted international diagnostic criteria of CD, UC or IBDU [1,2]. The healthy controls consisted of an age- and gender matched group that also underwent ileocolonoscopy (negative for IBD). This group did not show evidence or familial history of IBD or any other immune mediated disorders. Venous blood samples were drawn from all patients and healthy controls. Sera were immediately frozen at -20°C. In patients, the first sample was obtained at baseline, immediately after endoscopic evaluation (before treatment was initiated). Thereafter a second sample was collected at an arbitrary visit to our hospital, with an interval of at least three months from the first sample.

Classification and definitions

Disease phenotype (location and behavior), at presentation and at latest follow-up, was assessed according to the Montreal classification [28]. Endoscopic severity of the disease in CD was assessed by the simple endoscopy score for CD (SES-CD) and for UC by the Mayo score [29]. Clinical disease activity was assessed using the Harvey Bradshaw index (HBI) for CD and Montreal classification for UC [28,30].

Overall use- and response to medication was recorded. In general, therapeutic management of IBD after diagnosis was done according to the step-up approach, as recommended by the Dutch guidelines [31]. In UC patients, topical and/or oral 5-ASA was initiated as induction therapy. In case of severe pancolitis or when induction with 5-ASA treatment failed, systemic steroids were initiated. In CD patients, systemic steroids were started as induction therapy. In CD patients this was usually followed by the initiation of maintenance treatment (thiopurine derivates or methotrexate), especially in case of steroid-dependent disease. Anti-TNF therapy was initiated in both UC and CD in case of steroid refractory or steroid-dependent disease,

usually in combination with a thiopurine derivate. When disease did not respond to conventional treatment, resective surgery was considered. During relapse, escalation of maintenance treatment was considered. Surgery was defined as the resection of part of the bowel (resective) or abscess drainage or fistula operations (other).

At first serum sampling all IBD patients presented with active disease. At second serum sampling patients were grouped according to disease exacerbation or remission. Remission was defined as the disappearance of clinical symptoms after induction treatment (HBI<5/ Montreal=S0) at general physician's assessment in combination with endoscopic remission (SES-CD<4/Mayo=0) and/or normalization of radiological examination and/or decreasing parameters of inflammation like CRP or fecal calprotectin. Disease exacerbation was defined as occurrence of symptoms (HBI>4/Montreal>S0) combined with either increased fecal calprotectin levels and/or abnormalities on imaging and/or endoscopic activity (SES-CD>3/ Mayo>0), resulting in escalation of medical treatment or surgical intervention.

For the purpose of the study, severe disease outcome in CD was defined as the occurrence of penetrating or stricturing disease, perianal disease with the need for anti-TNF initiation and/ or surgical intervention and CD related resective surgery. In UC, severe disease was defined as step-up of treatment when 5-ASA and/or steroids failed in achieving and/or maintain remission, thus initiation of thiopurines or anti-TNF or Vedolizumab therapy or the need for a colectomy. These conditions were compared to the presence of serological antibodies.

Serological assays

Serum was separated from blood by centrifugation and kept frozen at -20 °C until analysis was performed. PANCA was determined by indirect immunofluorescence (EUROIMMUN Granulocyte Mosaic 13 slides) according to the manufacturers' protocol using a 1:10 serum dilution. PANCA was classified as positive or negative. Commercially available kits of enzyme-linked immunosorbent assays (ELISA) were used to measure the expression of the following antibodies in all sera: *g*ASCA IgA, ACCA IgA, ALCA IgG and AMCA IgG (Glycominds LTD, Lod, Israel). The laboratory technician was blinded for clinical phenotype and disease course; the clinical investigators had no access to antibody results at the time of clinical data acquisition. The assays were tested according to the manufacturers' protocol. Results were expressed as arbitrary ELISA units/milliliter (EU/mL), a concentration relative to a calibration serum sample. Manufacturers' cutoff levels were used: *g*ASCA IgA (positive>50EU/mL), ACCA IgA (positive>90EU/mL), ALCA IgG (positive>60EU/mL), AMCA IgG (positive>100EU/mL).

Inter- and intra-assay precision errors were controlled by 8 sera with different antibody profiles that were repeatedly tested in every run (n=3). The relative deviation from the mean for *g*ASCA IgA, ACCA IgA, ALCA IgG and AMCA IgG were 11%, 17%, 23% and 26%respectively.

Statistical analysis

Antibody results were displayed as serum concentration (anti-glycan antibodies; quantitative) and positive/negative (anti-glycan antibodies and pANCA; qualitative). The Shapiro-Wilk test was used to test normality. Categorical variables were expressed as numbers of patients and percentages and compared with Fisher's exact test. Continuous variables were expressed as median with its interquartile range (IQR) and compared with Mann-Whitney U test. Kruskal-Wallis analysis was used followed by the Dunn test. Multivariate analyses were performed using binary logistic regression to identify independent predictors of seropositivity for each individual marker and disease outcome corrected for age at diagnosis, gender, disease location and disease phenotype. Spearman's correlation coefficients were used to assess the correlation of baseline antibody concentration and status with disease activity scores and with serial measurements. Paired data over time were compared using the Wilcoxon matched-pairs signed-rank test for antibody concentration and McNemar's test for antibody status. We computed receiver operating characteristic (ROC) curves, which yielded an area under the curve (AUC), and defined the optimal cutoff value using the Youden's index. Sensitivity, specificity, positive and negative predictive values were calculated to assess the predictive power of anti-glycan antibodies and pANCA to differentiate between CD and UC and between CD and healthy controls (HC). SPSS statistics for Windows (version 22.0; IBM Corp, Armonk, NY, USA) and GraphPad Prism (GraphPad Software version 7.0, La Jolla, CA, USA) were used to analyze the data. Statistical significance was defined as a *P*-value less than 0.05.

Ethical considerations

Written informed consent was obtained from each participating patient before any study-related procedure was performed. The procedures were performed in accordance with the Declaration of Helsinki. The regional medical ethics committee approved the study protocol.

RESULTS

Patient characteristics

A total of 120 newly diagnosed IBD patients were included for baseline serologic measurement, of which 86 were classified as CD, 29 as UC and five as IBDU. The median follow-up duration was 27 months (IQR 14-59). Demographic and baseline characteristics can be found in table 1. Nineteen healthy controls were included. There were no statistical significant differences between the different patient groups and healthy controls for age or gender. CD patients had higher baseline CRP levels compared to healthy controls and to UC patients (both P=0.0001). IBD patients had higher baseline calprotectin levels compared to healthy controls (P=0.0001 for CD and UC, P=0.017 for IBDU).

Table 1. Baseline characteristics.

	CD (n=86)	UC (n=29)	IBDU (n=5)	HC (n=19)
Median age at diagnosis in years (IQR)	27 (22-41)	30 (24-39)	23 (22-45)	35 (26-47)
Female, n (%)	58 (67%)	18 (62%)	2 (40%	15 (79%)
Symptoms before diagnosis in months				-
0-3	26 (30%)	13 (45%)	3 (60%)	
3-6	18 (21%)	14 (48%)	1 (20%)	
>6	42 (49%)	2 (7%)	1 (20%)	
Median follow-up period in months (IQR)	30 (16-64)	20 (12-51)	20 (10-65)	-
Median time in months between first and second sample (IQR)	30 (12-50)	17 (12-29)	25 (9-49)	-
Family with IBD, n (%)	12 (14%)	9 (31%)	0 (0%)	0 (0%)
Smoking status at diagnosis				
• Never	41 (48%)	24 (83%)	3 (60%)	12 (63%)
• Current	38 (44%)	2 (7%)	1 (20%)	4 (21%)
• Ceased	7 (8%)	3 (10%)	1 (20%)	3 (16%)
CRP	19 (9-58)	5 (1-15)	2 (1-22)	4 (1-6)
Calprotectin	476 (238-1120)	381 (194-676)	1352 (525-1692	6 (0-15)
Median HBI score (IQR)	8 (6-13)	-	15 (9-22)	-
CD disease location (Montreal)		-		-
• Ileal (L1), n (%)	32 (37%)		-	
• Colonic (L2), n (%)	19 (22%)		5 (100%)	
• Ileocolonic (L3), n (%)	35 (41%)		-	
• + Upper GI (+L4), n (%)	22 (39%)		2 (50%)	
• + Perianal disease (+P), n (%)	13 (15%)		-	
CD disease behaviour (Montreal)		-		-
• Inflammatory, n (%)	67 (78%)		5 (100%)	
• Stricturing, n (%)	15 (17%)		-	
• Penetrating, n (%)	4 (5%)		-	
Median SES-CD score (IQR)	12 (8-21)	-	18 (10-21)	-
SES-CD severity		-		-
• Mild (4-10), n (%)	33 (38%)		1 (20%)	
• Moderate (11-19), n (%)	29 (34%)		2 (40%)	
• Severe (>19), n (%)	24 (28%)		2 (40%)	

Table 1. continued

	CD (n=86)	UC (n=29)	IBDU (n=5)	HC (n=19)
UC clinical score (Mayo)	-			-
• Remission, n (%)		0 (0%)	0 (0%)	
• Mild, n (%)		12 (41%)	1 (20%)	
• Moderate, n (%)		10 (34%)	2 (40%)	
• Severe, n (%)		7 (24%)	2 (40%)	
UC disease location (Montreal)	-			-
• Proctitis (E1), n (%)		6 (21%)	1 (20%)	
• Left-sided (E2), n (%)		8 (28%)	1 (20%)	
• Pancolitis (E3), n (%)		15 (52%)	3 (60%)	
UC endoscopic severity (Montreal)	-			
• Normal, n (%)		0 (0%)	0 (0%)	19 (100%)
• Mild, n (%)		7 (24%)	1 (20%)	
• Moderate, n (%)		16 (55%)	3 (60%)	
• Severe, n (%)		6 (21%)	1 (20%)	
Recurrence after first period of remission, n (%)	38 (46%)	21 (72%)	4 (80%)	
Highest treatment step during follow-up				-
• 5-ASA	3 (3%)	13 (45%)	0 (0%)	
• Oral steroids	10 (12%)	5 (17%)	1 (20%)	
• Immunosuppressants	41 (48%)	7 (24%	3 (60%)	
• Anti-TNF	23 (27%)	1 (3%)	1 (20%)	
• Resective surgery	9 (10%)	3 (10%)	0 (0%)	

IBD=inflammatory bowel disease, CD = Crohn's disease, UC = ulcerative colitis, IBDU = IBD unclassified, HC = healthy controls, n = number, IQR = interquartile range, HBI = Harvey-Bradshaw Index, SES-CD= simple endoscopic score for Crohn's disease

Antibodies at diagnosis for differentiating IBD

Seropositivity of any antibody at diagnosis was detected in 43% of CD patients, 48% of UC patients, 20% of IBDU patients and 5% of healthy controls (table 2). In CD patients this was mainly attributed to the presence of ASCA (23%), followed by positive ALCA (13%), AMCA (9%), ACCA (6%) and pANCA (5%). Patients with UC were mainly positive for pANCA (45%), followed by ASCA (3%) and AMCA (3%) with no ALCA or ACCA present in the serum. One IBDU patient was seropositive for pANCA, with no detectable anti-glycan antibodies. One healthy control was positive for ACCA, all other antibodies tested negative in controls.

Differentiating IBD patients from healthy controls by the use of antibody status could be done with pANCA in UC patients (P=0.0001) and with ASCA in CD patients (P=0.012). ASCA seropositivity could differentiate CD from UC (P=0.011), ALCA positivity was also associated with CD (P=0.034), while pANCA was associated with UC (P=0.0001) (see table 2). Any positive

anti-glycan with negative pANCA could differentiate CD patients (33/86) from UC patients, who were mainly anti-glycan negative/pANCA positive (13/29 patients, *P*=0.0001). We analyzed the predictive power of the different antibodies, with the previously defined cutoff levels, in differentiating CD from UC patients and CD patients from healthy controls (table 3 and 4, respectively). Predefined cutoff levels showed low accuracy, with high specificity but low sensitivity. Therefore, we constructed ROC curves and used the Youden's index to define the optimal cutoff values and their accuracy in our patient group (figure 1, table 3 and 4). Optimal cutoff levels differed considerably from the predefined cutoff levels. The most accurate single marker in differentiating CD from UC was ASCA (AUC = 0.820) and the combination of ASCA and pANCA added only slightly to this accuracy (AUC = 0.891). Using all anti-glycans and pANCA did not improve accuracy more (AUC=0.887). ASCA and ALCA were the most accurate single markers for the differentiation of CD patients from HC (AUC 0.790 and 0.773, respectively) and a combination of all anti-glycans improved accuracy to an AUC of 0.851.

After multivariate analysis, with correction for age, gender, smoking habit and other antibodies, pANCA status (*P*=0.003) and the combination of ASCA and pANCA status (*P*=0.004), were able to discriminate between UC and CD, whilst other antibodies were not independently associated.

Serological status did not correlate with clinical (HBI/Montreal) and endoscopic (SES-CD/Mayo) disease activity scores at diagnosis. A negative correlation was found for CRP with pANCA status (rho=-0.285, *P*=0.003). In UC patients, calprotectin levels at diagnosis correlated strongly with pANCA status (rho=0.545, *P*=0.019). No such correlations were found in CD patients.

Table 2. Serological results at diagnosis.

	CD (n=86)	UC (n=29)	IBDU (n=5)	HC (n=19)	*P*-value
Markers, any positive, n (%)	37 (43)	14 (48)	1 (20)	1 (5)	CD-UC 0.527, **UC-HC 0.005, CD-HC 0.001,** IBDU-HC 0.380
gASCA positive, n (%)	20 (23)	1 (3)	0 (0)	0 (0)	**CD-UC 0.011,** UC-HC 0.604, **CD-HC 0.012**
AMCA positive, n (%)	8 (9)	1 (3)	0 (0)	0 (0)	CD-UC 0.284, UC-HC 0.604, CD-HC 0.190
ALCA positive, n (%)	11 (13)	0 (0)	0 (0)	0 (0)	**CD-UC 0.034,** CD-HC 0.098, CD-UC 0.227, UC-HC 0.396, CD-HC 0.703
ACCA positive, n (%)	5 (6)	0 (0)	0 (0)	1 (5)	**CD-UC 0.0001, UC-HC 0.0001,** CD-HC = 0.444
pANCA positive, n (%)	4 (5)	13 (45)	1 (20)	0 (0)	

IBD = inflammatory bowel disease, CD = Crohn's disease, UC = ulcerative colitis, IBDU = IBD unclassified, HC = healthy control, n= number, IQR = interquartile range, statistically significant *P*-values are shown in **bold**

Table 3. Predictive power of antibodies in differentiating between CD and UC.

	Cut-off level CD vs UC	Youden's index	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
ASCA predefined	50 EU	0.198	23%	97%	95%	30%
ASCA optimal	10 EU	0.606	71%	90%	95%	51%
ALCA predefined	60 EU	0.128	13%	100%	100%	28%
ALCA optimal	20 EU	0.340	62%	72%	87%	39%
ACCA predefined	90 EU	0.058	6%	100%	100%	26%
ACCA optimal	53 EU	0.210	28%	93%	92%	30%
AMCA predefined	100 EU	0.047	10%	97%	90%	27%
AMCA optimal	36 EU	0.353	70%	65%	86%	42%
pANCA	-	0.402	45%	95%	76%	84%
Combination all anti-glycans and pANCA[1]	0.2923933	-0.642	85%	79%	92%	64%
Combination of ASCA and pANCA[2]	0.2815950	-0.666	91%	76%	92%	73%

[1]y=1.094 + ASCA*-0.038 + ALCA*-0.019 + ACCA*-0.013 + AMCA*-0.026 + pANCA*3.053
[2]y=-0.595 + ASCA*-0.056+ pANCA*2.460

Table 4. Predictive power of antibodies in differentiating Crohn's disease (CD) and healthy controls (HC).

	Cut-off level CD vs HC	Youden's index	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
ASCA predefined	50 EU	0.233	23%	100%	100%	22%
ASCA optimal	20 EU	0.512	51%	100%	100%	31%
ALCA predefined	60 EU	0.128	13%	100%	100%	20%
ALCA optimal	17 EU	0.551	71%	84%	95%	39%
ACCA predefined	90 EU	0.006	6%	95%	83%	19%
ACCA optimal	23 EU	0.206	84%	37%	86%	33%
AMCA predefined	100 EU	0.081	9%	100%	100%	20%
AMCA optimal	40 EU	0.277	59%	68%	89%	27%
Combination of anti-glycans[1]	0.8464560	0.654	70%	95%	98%	41%

[1]^y=-1.784 + ASCA*0.090 + ALCA*0.069 + ACCA*0.006 + AMCA*0.007

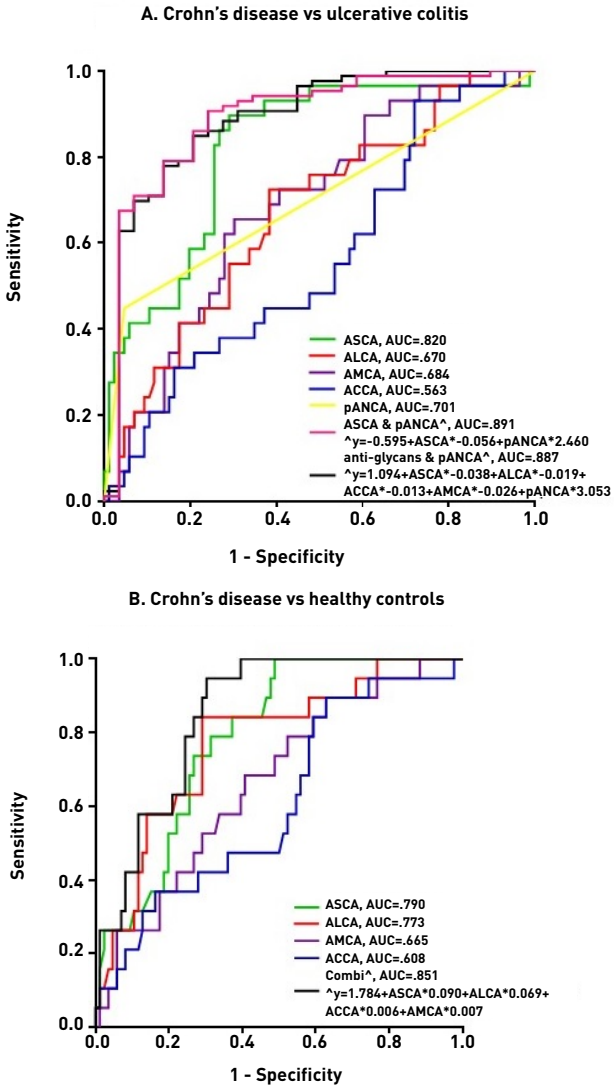

Figure 1. (a) Discrimination between CD and UC patients based on anti-glycan antibodies and pANCA. (b) Discrimination between CD patients and HC based on anti-glycan antibodies, combi = the combination of ASCA, ALCA, AMCA and ACCA.

Association of antibody status at diagnosis with disease phenotype and outcome in CD patients

At univariate analysis, no association was found for any of the antibodies with disease phenotype or -course. In CD patients with a follow-up duration of at least one year (n=70), severe disease developed in 22 patients (31%). Classification of severe disease was based on resective CD

related surgery (7/22), perianal disease with the need of anti-TNF initiation and/or surgical intervention (10/22) and penetrating or stricturing disease at last follow-up (5/22). There was no association between the baseline presence of antibodies and the development of severe disease. In this group 36 patients (51%) had at least one exacerbation after initial remission (relapsing disease), while 34 patients (49%) maintained remission. There was no association between the presence of antibodies at baseline and relapsing disease.

Association of antibody status at diagnosis with disease phenotype and outcome in UC patients

In UC patients there was no association of antibody status with any of the patient characteristics described in table 1, including disease location and endoscopic severity. In patients with a follow-up duration of at least one year (n=22), severe disease developed in nine UC patients (41%). This classification was based on colectomy (2/9), anti-TNF therapy (1/9) or thiopurine therapy after 5-ASA failure (6/9). Relapsing disease (at least one exacerbation after initial remission) occurred in 18 patients (81%) while 4 patients (18%) maintained remission. There were no associations with antibody status and severe disease outcome or relapsing disease in UC patients.

Serologic change over time

Serial serologic assessment was available in 71 IBD patients (49 CD, 18 UC and four IBDU, see table 5 and table 6), after a median follow-up duration of 26 months (IQR 13-47). A change of serum antibody status compared to initial assessment was observed for pANCA in 15% of IBD patients, for ASCA in 10%, for ALCA in 10%, for AMCA in 9% and for ACCA in 6% (table 6). Antibody concentrations were stable over time in most cases.

In 86-94% (antibody dependent) of CD patients anti-glycan antibodies and pANCA presence remained unchanged at serial measurement compared to diagnosis. However, AMCA, ACCA and ALCA concentrations did show a statistical significant change from baseline ($P<0.05$, table 5). There was a trend towards a stronger decrease of AMCA concentrations in CD patients using anti-TNF treatment at serial measurement ($P=0.092$) while other types of treatment did not affect the change in antibody concentrations.

At the moment of follow-up blood collection 33 CD patients were in remission while 16 patients experienced an exacerbation. There was a comparable follow-up duration for these two groups ($P=0.424$). Changes in the presence of antibodies were comparable between the two groups (antibodies $P>0.05$). ACCA and AMCA concentrations decreased from baseline values in both groups (ACCA: inactive $P=0.002$ and active $P=0.005$; AMCA: inactive $P=0.006$ and active $P=0.007$ respectively). However, there were some differences in the change of antibody concentration between the two groups. A trend towards a higher increase in ALCA concentrations from baseline to follow-up was observed in patients with inactive disease when compared to active disease ($P=0.055$ and $P=0.352$ respectively). ASCA concentrations decreased more in patients with inactive disease ($P=0.085$) than in patients with active disease ($P=0.642$).

Table 5. Serologic results at serial measurement, during follow-up

	FU CD (n=49)	FU UC (n=18)	FU IBDU (n=4)
Markers, any positive, n (%)	19 (39%)	7 (39%)	2 (50%)
gASCA positive, n (%)	7 (14%)	0 (0%)	0 (0%)
Change in gASCA level, median EU/ml (IQR)	-1 (-5;+2)	0 (-1;+1)	-1 (-3;0)
AMCA positive, n (%)	2 (4%)	0 (0%)	0 (0%)
Change in AMCA level, median EU/ml (IQR)	-11 (-30;-2)*	-1 (-15;+6)	+12 (-5;+27)
ALCA positive, n (%)	10 (20%)	0 (0%)	0 (0%)
Change in ALCA, median EU/ml (IQR)	+3 (-3;+12)*	+4 (0;+9)*	+3 (+2;+4)
ACCA positive, n (%)	2 (4%)	1 (6%)	0 (0%)
Change in ACCA level, median EU/ml (IQR)	-7 (-17;-1)*	-5 (-9;+3)	-4 (-8;+16)
pANCA positive, n (%)	3 (6%)	6 (33%)	2 (50%)

FU = follow-up, CD = Crohn's disease, UC = ulcerative colitis, IBDU = IBD unclassified, n= number, IQR = interquartile range

*change in level has a $P<0.05$ when compared to baseline

In UC patients, anti-glycan antibody status remained unchanged at follow-up, except for ACCA, which became positive in 6% of patients. PANCA remained stable in 67% of UC patients, while 17% became negative and 17% became positive at serial measurement. This change in pANCA presence correlated with disease activity at follow-up (rho= 1.000, P=0.01), as the three patients that became negative all achieved remission while the three patients that became positive all had an exacerbation at serial measurement. However, in patients with stable pANCA status (n=12) no correlation was found with disease activity at serial measurement.

In UC patients there was a trend towards a stronger decrease from baseline of ASCA concentrations when patients were using thiopurine medication at serial measurement (P=0.053). Other treatments did not affect the change in antibody concentrations in UC.

At the moment of follow-up blood collection ten UC patients were in remission while eight patients experienced an exacerbation. There was a comparable follow-up duration for these two groups (P=0.726). Antibody presence did not change in a different manner between the two groups (all P>0.05). Change in AMCA (P=0.799 and P=0.123) and ASCA (P=0.959 and P=1.000) concentrations was comparable between the two groups. However, ACCA concentrations decreased more from baseline to follow-up in patients with inactive disease (P=0.037) compared to patients with active disease (P=0.575), whereas ALCA concentrations increased from baseline to follow-up in patients with inactive disease compared to active disease (P=0.037 and P=0.401 respectively).

In IBDU no changes were observed for the anti-glycan antibodies, while 50% of pANCA status changed from negative to positive at follow-up.

Table 6. Changes in serological results from baseline to follow-up.

Marker	IBD (n=71)	CD (n=49)	UC (n=18)	IBDU (n=4)
ASCA				
No change	64 (90%)	42 (86%)	18 (100%)	4 (100%)
+ to –	5 (7%)	5 (10%)	-	-
- to +	2 (3%)	2 (4%)	-	-
McNemar *P*-value		0.453		
AMCA				
No change	65 (91%)	43 (88%)	18 (100%)	4 (100%)
+ to –	4 (6%)	4 (8%)	-	-
- to +	2 (3%)	2 (4%)	-	-
McNemar *P*-value		0.688		
ALCA				
No change	64 (90%)	42 (86%)	18 (100%)	4 (100%)
+ to –	2 (3%)	2 (4%)	-	-
- to +	5 (7%)	5 (10%)	-	-
McNemar *P*-value		0.453		
ACCA				
No change	67 (94%)	46 (94%)	17 (94%)	4 (100%)
+ to –	2 (3%)	2 (4%)	-	-
- to +	2 (3%)	1 (2%)	1 (6%)	-
McNemar *P*-value		1.000		
pANCA				
No change	60 (84%)	46 (94%)	12 (67%)	2 (50%)
+ to –	5 (7%)	2 (4%)	3 (17%)	-
- to +	6 (8%)	1 (2%)	3 (17%)	2 (50%)
McNemar *P*-value		1.000		

DISCUSSION

In this cohort of newly diagnosed untreated IBD patients 43% of CD and 48% of UC patients were seropositive for any antibody at baseline. This was mainly attributed to ASCA positivity in CD patients and pANCA positivity in UC, while newer anti-glycan antibodies (ACCA, ALCA and AMCA) were less prevalent and mostly found in CD. Anti-glycan antibodies and pANCA had a good accuracy for differentiating IBD from healthy controls and differentiating CD from UC. Overall, there was a good correlation between baseline and serial measurement of antibody presence, although concentration of antibodies did show some significant changes from baseline to follow-up, sometimes related to specific immunosuppressive therapy. A change in the presence of pANCA occurred in up to 34% of pANCA change in UC patients. This change

in pANCA presence in UC patients correlated with disease activity at serial measurement. No associations were found with disease phenotype, severity of disease activity or disease course.

The antibody prevalence in this study was relatively low compared to previous studies, where a prevalence of positive antibodies in IBD patients was described as followed: of 42-69% ASCA positivity in CD (23% in our study), 19-27% ALCA positivity in CD (here 13%), 12-28% AMCA positivity in CD (here 9%), 8-25% ACCA positivity in CD (here 6%) and pANCA positivity in UC patients of 41-73% (45% here) [9,20]. An explanation for these differences could be that we included patients at the time of initial diagnosis in a secondary care hospital, representing the real-world IBD population and not a referral-based population, probably including more complex patients. Supporting this explanation, the ASCA and pANCA prevalence in the present study are in line with a Norwegian population-based cohort study [32]. Cutoff values of anti-glycan antibodies have been established previously with ROC curve analysis to differentiate CD from UC [11]. This was done in a referral-based population of IBD patients with longstanding disease (mean duration of eight years for UC and nine years for CD) all under medication and up to 39% of patients with bowel surgery. This could also be an explanation for the lower prevalence found in our cohort.

A predictive role for serological antibodies in UC patients at diagnosis or during follow-up cannot be supported by our results, as few patients were positive for other antibodies than pANCA and no associations were found with disease course. Similarly, in CD patients no associations were found between antibody presence at baseline and disease course. Previously described associations with age, disease location (ileal), perianal disease and future surgery could not be confirmed in our patient cohort [20]. In other immune mediated diseases, serum autoantibodies can be used to monitor disease activity, where the autoantibody level can be associated to disease remission and treatment response [22-27]. In autoimmune hepatitis autoantibodies are known to fluctuate before and after immunosuppressive therapy and persisting high titers are associated with disease activity [22]. Recently, it was shown in early rheumatoid arthritis that anti-citrullinated protein antibodies (ACPAs) could disappear from the circulation upon antirheumatic therapy [23]. ACPA and rheumatoid factor levels also decrease after anti-TNF therapy, significantly more in responders [24]. ANCA titers are routinely checked in diagnosis and follow-up of vasculitis [25]. In systemic lupus erythematosus, elevated anti-double-stranded DNA antibody concentrations precede disease flares [33,34]. In celiac disease disappearance of autoantibodies indicates treatment adherence [26,27].
In our study, disease activity scores (both clinical and endoscopic) in IBD patients at diagnosis did not correlate with presence or concentration any of the antibodies in serum. However, all patients had some degree of disease activity at diagnosis and therefore our patient cohort at baseline is more homogeneous than when comparing patients with active and inactive disease. During follow up the anti-glycan antibody concentration showed clear variety between active

and inactive disease, while the presence of antibodies at follow up did not differentiate between active or inactive disease. Anti-glycan antibodies are antibodies to cell wall carbohydrate epitopes found in bacteria and fungi [20]. Their presence therefore reflects increased intestinal permeability but might also reflect a loss of tolerance towards microbiota [20]. Their involvement in the IBD pathogenesis is disputable by their low prevalence in patients and their largely unaffected status by inflammatory burden (disease activity). However, the heterogeneous prevalence of anti-glycan antibodies in CD patients underlines the heterogeneity of this group of patients and supports the idea of a multifactorial pathogenesis in CD.
On the other hand, in 34% of UC patients, change in pANCA status correlated with disease activity at follow-up measurement, as patients that became positive all had an exacerbation and patients that became negative all achieved remission at that moment. Therefore, there might be a role for monitoring pANCA in UC patients for assessment of disease activity. This was also underlined by the strong correlation of baseline pANCA status with calprotectin values. Recently it has been shown in UC patients that pANCA positivity declined after treatment compared to baseline [18]. PANCA is directed against a protein of the nuclear envelope of neutrophils [35]. In line with the fact that UC has a more homogeneous disease presentation than CD, until now only one antibody (pANCA) seems relevant in this disease. Even though pANCA positivity in UC is not 100%, it might still play a role in the pathogenesis or it might represent a bystander effect of chronic inflammation.

PANCA and anti-glycan antibodies in serum could be helpful to categorize IBDU patients as being CD or UC. Three of five IBDU patients in our cohort were pANCA positive (one at diagnosis, two at serial measurement), without any anti-glycan antibodies present. It was previously found that ASCA-/pANCA+ predicted UC in 64% of IBDU patients, whereas ASCA+/pANCA- was more helpful as it predicted CD in 80% of cases [8]. At present, with a median follow-up of 20 months after diagnosis (IQR 10-65), none of the three pANCA positive IBDU patients could (yet) be diagnosed as having UC nor as having CD.

We showed that, even though there was an overall stable presence of anti-glycan antibodies, there is inter-individual variation of antibody concentration and status over time. These findings are in line with previous studies, in patients with longstanding IBD, showing marked concentration changes over time with status change to a lesser extent [21,36,37]. It depends on which percentage of status change would be generally acceptable, to clearly state (in)stability over time. ALCA concentrations increased more in IBD patients in remission at follow-up blood collection, while AMCA and ACCA concentrations decreased over time, irrespective of disease activity at the moment of follow-up blood collection. Furthermore, there was a trend for an association between thiopurine use and anti-TNF treatment at second blood collection with change in antibody concentration. Thiopurine medication induced a stronger decrease of ASCA in UC patients while anti-TNF treatment in CD patients induced a stronger decrease of

AMCA concentrations. These findings imply the possibility of confounding factors by specific immunomodulatory treatment on antibody status. This should be taken into account when studying antibodies in IBD patients under treatment. Furthermore, it would be interesting to study these antibodies into more detail in a larger patient cohort prior to initiation of medication and evaluating response to treatment.

We identified several limitations of this study. As longer follow-up duration (>10 year) is generally accepted as ideal to determine disease outcome in IBD patients, we were limited by a shorter follow-up duration and therefore definite conclusions on the relation between antibodies and development of complications cannot be drawn. The low number of IBDU patients precludes us from drawing robust conclusions about the utility of serological antibodies in this patient group. However, given the high AUC (0.891) of the combination between ASCA and p-ANCA in discriminating between CD and UC, these two antibodies can have an added value to the primary diagnostic work-up in newly diagnosed IBDU patients.
Strengths of this study were that it was performed in a secondary care center with access to the full range of IBD phenotypes (no selection bias) and prospective inclusion of solely newly diagnosed IBD patients. All patients had endoscopic confirmed inflammation and were untreated at baseline, followed by serial measurements that were performed to determine stability over time.

CONCLUSION

In conclusion, this population-based study confirms diagnostic value of analyzing ASCA and pANCA for differentiating IBD. Other anti-glycan antibodies were found in low prevalence, almost exclusively in CD patients. The anti-glycan antibody profile remains unchanged during follow up, with a status change in only 6-14% of CD patients, irrespective of their disease activity. The pANCA status change which occurred in 34% of UC patients, correlated with disease activity at serial measurement and could be added as a biomarker to assess disease activity. Although antibody presence was quite stable over time, antibody concentrations did fluctuate significantly, sometimes related to specific immunosuppressive therapy. In the present study, there is no evidence found for a predictive or prognostic role of serological antibodies in adult IBD patients.

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7

Candidate serum markers in early Crohn's disease: predictors of disease course

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ABSTRACT

Background and Aims

More than half of patients with Crohn's disease (CD) develop disease complications requiring aggressive medical therapy or surgery over time. However, predicting disease course and treatment response remains difficult. We therefore identified distinctive serum analytes associated with disease activity and course in newly diagnosed, untreated patients at presentation and during their follow-up.

Methods

In a pilot study, a multiplex immunoassay analysis on 36 markers was performed on serum from 20 CD patients at the time of primary diagnosis following endoscopic evaluation. The 12 most potent markers associated with disease activity, phenotype and course were analysed in a consecutive cohort of 66 CD patients at diagnosis and follow-up (n=39). A healthy control group (n=20) was included as a reference.

Results

CD patients had higher baseline levels of sTNF-R2 (P=0.001), sIL-2R (P=0.0001) and MMP-1 (P=0.001) compared with healthy controls. Serial measurements revealed that these three analytes dropped statistically significantly from baseline level during remission and were high during exacerbation. Great decline of sTNF-R1 levels was found during remission with 6.7 fold lower levels than in healthy controls (P=0.015). Patients who did not respond to initial prednisone treatment had higher baseline levels of sTNF-R2 (P=0.001). Patients experiencing relapses during follow-up had lower baseline sTNF-R2 and VCAM levels compared with patients with long-lasting remission.

Conclusions

In a large cohort of newly diagnosed untreated CD patients we identified candidate serum markers (sTNF-R1, sTNF-R2, sIL-2R and MMP-1) associated with disease activity. Furthermore, sTNF-R2 was associated with prednisone response and, together with VCAM, with long-lasting remission.

INTRODUCTION

Crohn's disease (CD) is an idiopathic chronic inflammatory disease, primarily affecting the gastrointestinal tract and belonging to the group of inflammatory bowel diseases (IBD). CD is characterised by lifelong episodes of relapsing-remitting disease and covers a wide spectrum of disease phenotypes. Disease behaviour at diagnosis consists of uncomplicated mucosal inflammation in 70-80% of patients, whereas 20-30% have already developed complications with stricturing or penetrating disease [1,2]. At presentation of the disease, younger age (<40), perianal or stricturing disease, involvement of the upper gastrointestinal tract, penetrating disease and smoking are clinical determinants associated with a complicated disease course [3,4]. Approximately 50% of CD patients are expected to develop disease complications over time requiring aggressive medical therapy such as biologics, immunomodulators or surgery. Disease monitoring, on which clinical decisions depend, include clinical scores (often inaccurate [5]), radiological techniques, endoscopy (including histological analysis) and faecal calprotectin. Despite clinical determinants and disease monitoring, it is difficult to predict disease course and response to therapy in clinical practice. Therefore, treatment is based on generic principles, without much focus on the individual patient. Reliable predicting indicators (biomarkers) on disease course and response to therapy would help in focusing on fast and accurate therapy in the individual patient and prevent administration of potentially toxic and expensive medication to nonresponsive patients [6]. Some efforts in this respect, have been made in a study that identified $CD8^+$ T cell transcriptional signatures associated with frequently relapsing disease in IBD and in a study on serological antibodies in IBD [7,8].

The involvement of a disrupted immune system in initiation and progression of CD has been established, but the driving immune regulatory factors involved in the early stages of CD are still poorly understood. It has been suggested that CD originates as a result of a dysregulated immune response to gut bacteria, characterised by the infiltration of inflammatory cells derived from the circulation into the gut mucosa of a genetically susceptible host [9]. In a previous study, we focused on the characterisation of the lymphocytic infiltrate in colonic biopsies and identified a subpopulation of naive T-cells that was associated with the clinical phenotype at presentation [10]. In addition, the local production of cytokines upon stimulation of the mucosal biopsies was related to the infiltrated T cell subpopulations and the clinical presentation. This local release of cytokines and the expression of adhesion molecules and cytokine receptors are thought to contribute to recruitment of the inflammatory cells and perpetuation of local inflammation [11]. The localised immunological process in the gut most likely has systemic reflections, as CD is known to be a systemic disease with extra-intestinal manifestations. Therefore, the primary aim of this study was to identify distinctive serum analytes associated with disease activity in newly diagnosed, untreated CD patients at presentation and during follow-up. Our secondary aim was to study the correlation of these serum analytes with disease phenotype and course.

MATERIALS AND METHODS

Patient selection and sample collection

An initial analysis was performed in a pilot cohort of 20 newly diagnosed, adult CD patients. We selected this cohort based on clinical- and mucosal immunological phenotype as presented earlier [10]. Subsequent analysis was performed in a consecutive cohort of CD patients (n=66) and an age- and sex-matched healthy control group (n=20). Patients were recruited before initial diagnostic ileocolonoscopy, at the outpatient clinic of the Department of Gastroenterology and Hepatology at Rijnstate Hospital, Arnhem, The Netherlands. After clinical, endoscopic, histopathological and, when indicated, radiographic evaluation, all patients met the accepted international diagnostic criteria of CD [12].

Disease phenotype (location and behaviour) was assessed according to the Montreal classification at primary diagnosis and at latest follow-up [13]. The Harvey-Bradshaw Index (HBI) was used to score clinical symptoms. The simple endoscopy score for CD (SES-CD) was used to assess endoscopic disease severity [14]. Mild endoscopic disease activity was defined as a score of 4-10, moderate activity as 11-19 and severe activity as ≥20 [15].

In general, therapeutic management of CD after diagnosis was done according to the step-up approach [16]. Accelerated use of antitumor necrosis factor (TNF) therapy was occasionally initiated, in case of complex perianal disease, in addition to surgical treatment. In accordance with the step-up approach, systemic steroids were started as induction therapy. This was usually followed by the initiation of maintenance treatment (mainly thiopurines). In case of steroid-dependent, -refractory or -intolerant disease, anti-TNF therapy was initiated, usually in combination with thiopurines or methotrexate. If disease did not respond to conventional treatment, surgery was considered. In case of relapse, escalation of maintenance treatment was considered. Remission was defined as disappearance of clinical symptoms after induction treatment (HBI<5): general physician's assessment in combination with endoscopic remission (SES-CD<4) and/or normalization of imaging and/or decreasing parameters of inflammation like C-reactive protein (CRP) and/or faecal calprotectin. Relapse was defined as occurrence of new symptoms (HBI>4) after achieving clinical remission, combined with either increased faecal calprotectin levels and/or abnormalities on imaging and/or endoscopic activity (SES-CD>3), resulting in escalation of maintenance treatment or surgical intervention. Disease course included any of the following parameters: initial steroid response, long-lasting remission (no relapses during follow-up), progression to stricturing, penetrating or perianal disease, initiation of anti-TNF during follow-up or surgery during follow-up. Venous blood samples were drawn following endoscopic evaluation at primary diagnosis, before any immunomodulating treatment was initiated. Serum samples were coded and stored at -20°C until analysis. Follow-up blood samples were collected in 39 patients, either in remission or during an exacerbation.

Multiplex immunoassay

A total of 36 cytokines, chemokines, growth factors, soluble receptors and other mediators (hereafter denoted by 'analytes') were selected (see figure 1), based on their relevance in CD and other (auto)inflammatory diseases [17-21]. The following panel of analytes was tested in the pilot cohort: IL-1RA, IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-13, IL-17, IL-18, IL-21, IL-23p19, IL-33, IL-37, TNF-α, IFN-γ, CCL-2/MCP-1, CCL-3/MIP-1α, CCL-4/MIP-1β, CCL-5/RANTES, CCL-19/MIP-3β, CXCL-8/IL-8, CXCL-10/IP-10, CXCL-13/BLC, GM-CSF, EGF, VEGF, sICAM, sVCAM, sTNF-R1, sTNF-R2, sIL-2R/sCD25, sIL-6R/sCD126, MMP-1, MMP-3, MMP-9, S100A8/MRP8. Candidate analytes that showed to be associated with clinical phenotype at diagnosis, endoscopic disease severity at diagnosis, and disease course (table 1; and supplementary table 1 and 2) in the pilot cohort were selected and subsequently analysed in the consecutive CD patient cohort and healthy controls.

Multiplex immunoassays were performed at the MultiPlex Core Facility of the Laboratory of Translational Immunology (UMC Utrecht, The Netherlands) using an in-house validated platform (ISO9001). In brief, color-coded magnetic beads (MagPlex Microsperes, Luminex, Austin, Texas) were conjugated to analyte-specific antibodies and incubated with standard dilutions or sample for 1 h during continuous shaking in the dark. Samples were pre-treated with HeteroBlock (Omega biologicals, Inc., Bozeman, Montana, USA) to prevent interference by binding of heterophilic antibodies. Plates were washed (Bio-Plex Pro II Wash Station; Bio-Rad, Hercules, California, USA) and a corresponding cocktail of biotinylated detection antibodies was added followed by streptavidin-phycoerythrin (PE) incubation. Fluorescence intensity of PE was measured using a Flexmap 3D system (Luminex) and analysed using BioPlex Manager Software (version 6.1; Bio-Rad, USA) using five-parameter curve fitting. Undetectable analyte results in CD patients were replaced by the lowest measured result of the patient group divided in half. This surrogate value was always below the lower limit of detection.

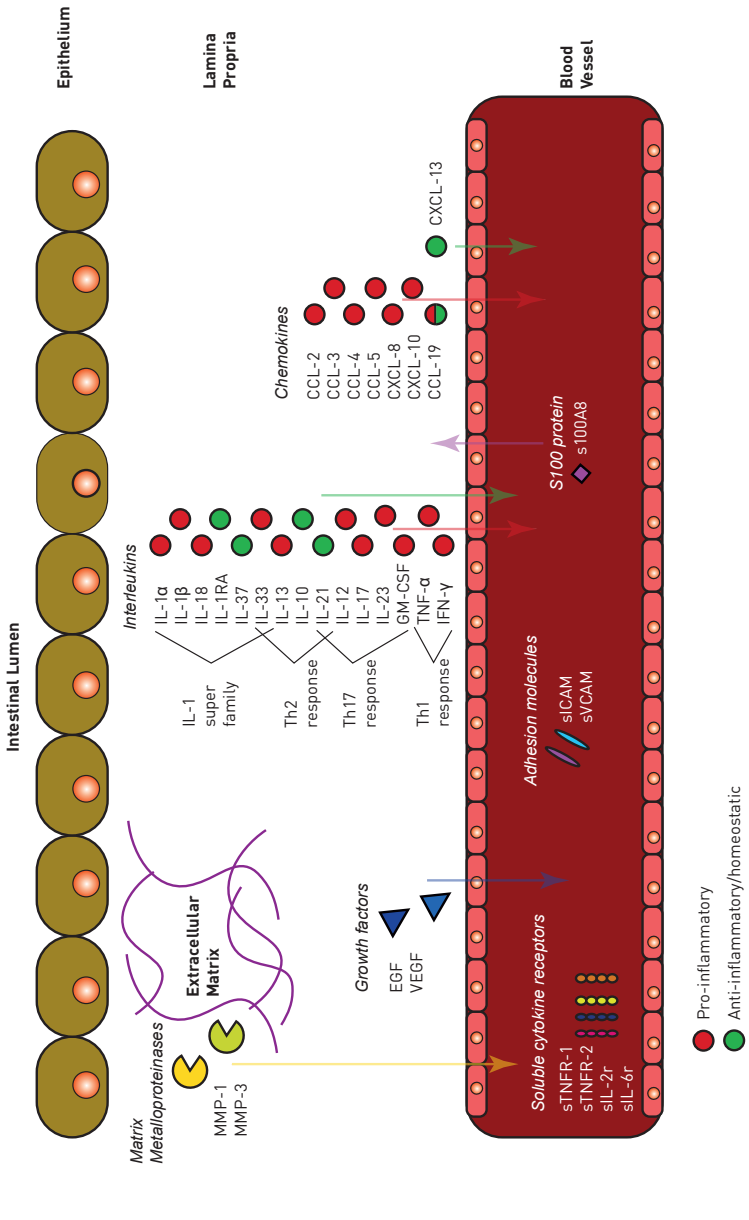

Figure 1. Main sites of action in the intestine and circulation of the measured serum analytes.

Table 1. Results of serum analytes associated with clinical phenotype, endoscopic severity and disease-course in the pilot cohort of 20 Crohn's disease patients. Composed of results shown more extensively in supplementary table 1 and 2.

Analyte	Clinical phenotype at diagnosis	*P*-value	Endoscopic disease severity at diagnosis	*P*-value	Disease course	*P*-value
sTNF-R1	↑ in patients with L2 phenotype	L2 vs. L1 0.026				
sTNF-R2	↑ in patients with L2 phenotype	L2 vs. L1 0.026	↑ when 10<SES-CD<19	Moderate vs. mild 0.036		
sIL-2R	↑ in patients with L2 phenotype	L2 vs. L1 0.008			↑ initial steroid non-responder	0.032
sIL-6R					↓ in patients without need of anti-TNF	0.009*
S100A8	↑ in patients with L2 phenotype	L2 vs. L3 0.022				
IL-37	↑ in patients with +P	0.033				
MMP-1			↑ when SES-CD>19	Severe vs. mild 0.039	↑ relapsing disease	0.046
MMP-9	↓ in patients with +P	0.025				
VCAM	↓ in patients with +P	0.025				
CCL-5					↑ relapsing disease	0.007
CCL-19					↑ initial steroid non-responder	0.003
CXCL-13					↓ in patients without need of anti-TNF	0.035*

SES-CD = Simple Endoscopy Score for Crohn's Disease

*Follow-up duration statistically significantly different between compared groups

Statistical analysis

The Shapiro-Wilk test was used to test the normality of variables. As most variables were skewed, log transformation was applied. Non-parametric tests were used for all log-transformed variables. Continuous variables were described as median and interquartile range (IQR) and compared with Mann-Whitney U tests. Categorical variables were described as absolute frequencies and compared with Kruskal-Wallis analysis followed by Dunn test. Due to the exploratory nature of the pilot group, no adjustments were made to the *P*-value for multiple comparisons. In the consecutive cohort, we report *P* values corrected for multiple testing using the Holm-Bonferroni correction. We adjusted for age and gender when testing for association between analyses and disease phenotype as they are common confounders (although no association with age and gender was found in our study). Analyte values measured in blood samples at baseline and those collected during follow-up were compared using Wilcoxon matched-pairs signed-rank test. We computed receiver operating characteristic (ROC) curves, defined the best thresholds for the analytes using the Youden's index and calculated the corresponding sensitivity, specificity, positive and negative predictive value. SPSS statistics for Windows (version 21.0.; IBM Corp, Armonk, NY, USA) and GraphPad Prism (GraphPad Software version 7.0, La Jolla, CA, USA) were used to analyse the data. Statistical significance was defined as a *P*-value lower than 0.05.

Ethical Considerations

Written informed consent was obtained from each participating patient before any study-related procedure was performed. The procedures were performed in accordance with the Declaration of Helsinki. The regional medical ethics committee approved the study protocol.

RESULTS

Serum analytes in a pilot cohort of newly diagnosed, untreated, CD patients

The complete panel of 36 analytes (figure 1) was tested in a pilot cohort of 20 newly diagnosed CD patients (table 2). Many analytes (IL-1α, IL-1β, IL-10, IL-12p70, IL-13, IL-17, IL-23p19, IL-33, IL-37, TNF-α, IFN-γ and GM-CSF) were below the limit of detection (supplementary table 3). Twelve analytes were associated with disease phenotype or disease course (table 2 and supplementary table 1&2): IL-37, CCL-5/RANTES, CCL-19/MIP-3β, CXCL-13/BLC, sVCAM, sTNF-R1, sTNF-R2, sIL-2R/sCD25, sIL-6R/sCD126, MMP-1, MMP-9 and S100A8/MRP8, and followed up in a consecutive cohort of CD patients (n=66) and age- and sex-matched healthy controls (n=20).

Table 2. Patient characteristics.

	CD pilot (n=20)	CD (n=66)	CD FU (n=39)[b]	Controls (n=20)[c]
Median age (at diagnosis in years, IQR)	30 (21-40)	27.5 (22.0-43.3)	29 (22.0-44.0)	26.5 (23.0-46.0)
Female, n (%)	14 (70%)	45 (68%)	27 (69%)	16 (80%)
Symptoms before diagnosis in months				
• 0-3	6 (30%)	20 (31%)	13 (33%)	
• 3-6	6 (30%)	15 (23%)	10 (26%)	
• >6	8 (40%)	31 (47%)	16 (41%)	
Median clinical follow-up period in months (IQR)	53 (27-66)[a]	40 (13-60)[a]	51 (16-61)	
Median months until second serum sample (IQR)	-	-	33 (13-54)	-
Smoking status at diagnosis				
• Never	10 (50%)	36 (55%)	20 (51%))	
• Current	10 (50%)	25 (38%)	17 (44%)	
• Ceased	-	5 (8%)	2 (5%)	
Median HBI score at diagnosis (IQR)	10 (8-12)	8 (6-13)	8 (6-13)	
Disease location at diagnosis (Montreal)				
• Ileal (L1), n (%)	4 (20%)	25 (38%)	13 (33%)	
• Colonic (L2), n (%)	4 (20%)	12 (18%)	6 (15%)	
• Ileocolonic (L3), n (%)	12 (60%)	29 (44%)	20 (51%)	
• + Upper GI involved (+L4), n (%)	8 (40%)	16 (24%)	11 (28%)	
• + Perianal disease (+P), n (%)	5 (25%)	11 (17%)	6 (15%)	

Table 2. continued

	CD pilot (n=20)	CD (n=66)	CD FU (n=39)[b]	Controls (n=20)[c]
Disease behaviour at diagnosis (Montreal)				
• Inflammatory, n (%)	13 (65%)	48 (73%)	28 (72%)	
• Stricturing, n (%)	6 (30%)	14 (21%)	10 (26%)	
• Penetrating, n (%)	1 (5%)	4 (6%)	1 (3%)	
Median SES-CD score at diagnosis (IQR)	16 (10-27)	12 (8-21)	13 (8-20)	
SES-CD severity at diagnosis				
• Mild (4-10), n (%)	5 (25%)	27 (41%)	15 (39%)	
• Moderate (11-19), n (%)	8 (40%)	20 (30%)	14 (36%)	
• Severe (>19), n (%)	7 (35%)	19 (29%)	10 (26%)	
Any recurrence after first moment of remission, n (%)	12 (60%)	31 (47%)	23 (59%)	
Treatment during blood drawl				
No medication	20 (100%)	66 (100%)	7 (18%)	
Oral steroids			2 (5%)	
Immunosuppressants			19 (49%)	
Anti-TNF			4 (10%)	
Combination anti-TNF + immunosuppressant			6 (15%)	
Combination of steroid + immunosuppressant			1 (3%)	
Highest treatment step during follow-up				
• 5-ASA	-	1 (2%)	-	
• Oral steroids	1 (5%)	4 (6%)	-	
• Immunosuppressants	9 (45%)	35 (53%)	22 (56%)	
• Anti-TNF	7 (35%)	19 (29%)	12 (31%)	
•Resective surgery	3 (15%)	7 (11%)	5 (13%)	

CD = Crohn's disease, FU = follow-up, IQR = interquartile range, HBI = Harvey-Bradshaw Index, SES-CD = simple endoscopic score for Crohn's disease, GI = gastrointestinal tract, 5-ASA = 5-aminosalicylic acid.

[a] P<0.05 between CD pilot cohort and consecutive CD cohort, no statistical significant differences for any of the other characteristics between these groups.

[b] no statistical significant differences for all characteristics between follow-up and baseline cohort.

[c] no statistical significant differences between healthy controls and any of the CD cohorts.

Association of analyte levels with disease phenotype at diagnosis

Baseline characteristics of the consecutive cohort are shown in table 2. All MMP-9 levels were above standard range and were not included for further analyses. Compared to healthy controls, CD patients had higher baseline levels of sTNF-R2 (fold change 1.5, *P*=0.001), sIL-2R (fold change 1.8, *P*=0.0001) and MMP-1 (fold change 2.0, *P*=0.001) as shown in table 3.

Shorter symptom duration before diagnosis (0-3 months) was associated with higher baseline MMP-1 and sIL-2R levels compared with longer (>6months) symptom duration (*P*=0.006 and *P*=0.005 respectively). Gender, age, smoking status, disease behaviour, and the presence of perianal disease at diagnosis were not associated with baseline analyte levels (data not shown).

When looking at disease location, patients with ileocolonic disease had higher baseline values of CXCL-13 (0.036), MMP-1 (0.009) and sIL-2R (*P*=0.003), than patients with solitary ileal disease. After correction for age, gender and symptom duration, only sIL-2R remained significant (*P*=0.006, with *P*=0.092 for CXCL-13 and *P*=0.164 for MMP-1).

When comparing severe endoscopic disease activity (SES-CD>19) at diagnosis with mild endoscopic disease activity (SES-CD 4-10), higher concentrations of MMP-1 were found in patients with severe disease activity (*P*=0.018, figure 2a). However, after correction for age and gender, SES-CD severity and symptom duration were not independent factors associated with MMP-1 levels. SIL-2R was higher in severe endoscopic disease compared with both mild (*P*=0.009) and moderate disease (*P*=0.041, figure 2b). After correction for age, gender and symptom duration, this remained a significant independent factor (*P*=0.009). STNF-R2 serum concentrations were higher in patients with severe endoscopic disease activity compared with patients with mild disease activity (*P*=0.038, corrected for age and gender, figure 2c).

Table 3. Serum analyte values of Crohn's disease (CD) patients at baseline and during follow-up, compared with healthy controls.

	Baseline CD patients (n=66)	Follow-up, CD in remission (n=29)	Follow-up, CD during exacerbation (n=10)	Healthy controls (n=20)
IL-37 pg/ml	5 (below detection limit)	5 (below detection limit)	5 (below detection limit)	5 (below detection limit)
CCL-19 pg/ml	116 (89-151)	101[bc] (86-127)	160[ac] (129-203)	88 (65-138)
CXCL-13 pg/ml	35 (26-48)	28[bc] (21-39)	47[ac] (34-78)	28 (21-38)
sIL-2R pg/ml	190[a] (112-327)	99[bc] (33-179)	232[ac] (155-282)	106 (69-145)
sTNF-R1 pg/ml	4236 (1696-5909)	557[abc] (313-1864)	2828[bc] (1160-4172)	3742 (366-5599)
sTNF-R2 pg/ml	1923[a] (1593-2320)	1388[b] (1235-1587)	1661 (1294-2035)	1277 (1036-1933)
S100A8 pg/ml	4762 (3106-8784)	2137[b] (1454-4916)	2361 (1860-3872)	3812 (1744-5974)
CCL-5 ng/ml*	112 (67-168)	82[b] (41-12)	91 (64-134)	99 (59-148)
VCAM ng/ml*	1549 (1197-1927)	1525 (1227-1759)	1355 (1278-1972)	1356 (1152-1830)
MMP-1 ng/ml*	47[a] (22-85)	24[b] (18-55)	34[ab] (27-50)	23 (12-32)
MMP-9 pg/ml	extrapolated above standard range	extrapolated above standard range	extrapolated above standard range	extrapolated above standard range
sIL-6R ng/ml*	21 (18-26)	19[b] (14-22)	22 (18-30)	22 (17-25)

[a] *P*-value <0.05 compared to healthy controls, [b] *P*-value<0.05 compared to baseline values [c] *P*-value <0.05 inactive versus active,

*Please note that this unit (ng/ml) differs from other analyte units in this table (pg/ml)

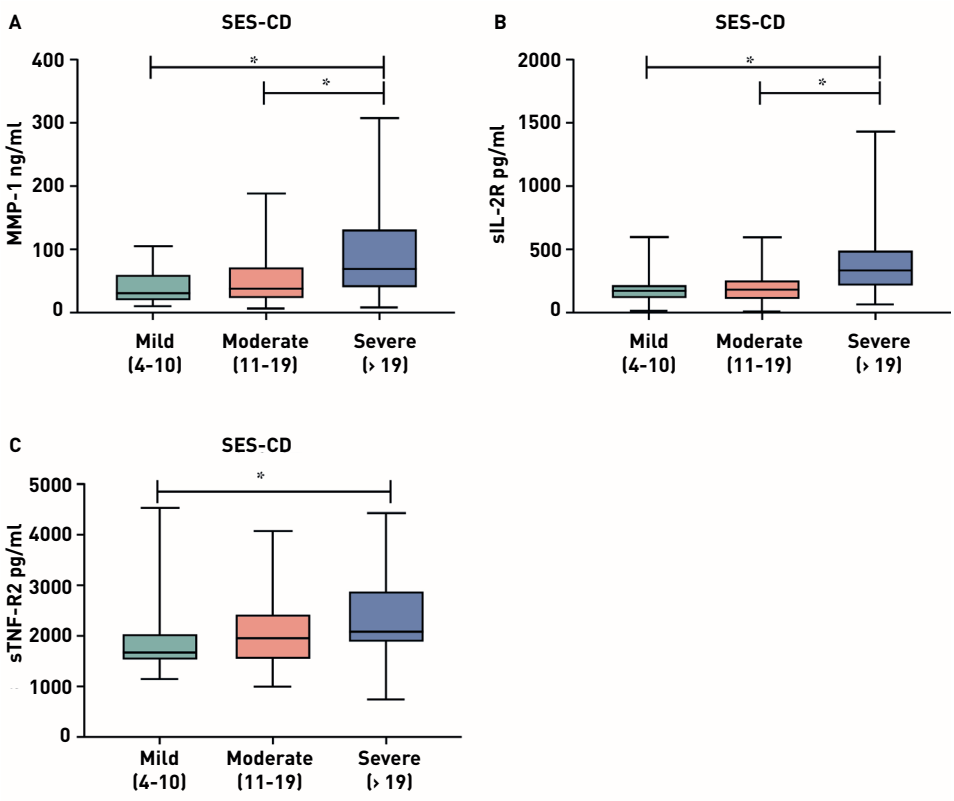

Figure 2. Serum analyte values at diagnosis (baseline) in Crohn's disease (CD) patients showing different groups of endoscopic disease activity based on SES-CD.

A. values of MMP-1 in ng/ml, B values of sIL-2R in pg/ml and C values of sTNF-R2 in pg/ml. *P<0.05. SES-CD = Simple Endoscopy Score for Crohn's disease; MMP = matrix metalloproteinase.

Association of analyte levels with disease course

Patients who did not respond to prednisone at initial treatment (n=9, 24%) had 1.5-fold higher pre-treatment levels of sTNF-R2 compared with steroid responders (n=28, 76%) (*P*=0.001). In the non-responding group, more patients needed initial stepping up to anti-TNF treatment (*P*=0.002) or resective surgery (*P*=0.011).

When examining patients with a follow-up longer than one year (n=51, median follow-up 51 months (IQR 23-63)), 29 patients (57%) suffered from at least one relapse after initial remission, whereas 22 (43%) patients maintained remission. There was no statistical significant difference in follow-up duration. Patients with relapsing disease had lower baseline sTNF-R2 levels (1821 pg/ml (IQR 1546-2044) compared with patients in long-lasting remission with 2073 pg/ml (IQR 1834-2661), *P*=0.019), independently associated after correction for SES-CD, age and gender.

They also had lower VCAM levels with 1494 ng/ml (IQR 1252-1735) compared with 1831 ng/ml (IQR 1375-2475), *P*=0.024. Baseline analyte values were not associated with development of perianal disease, disease behaviour, initiation of anti-TNF medication, or surgical intervention (data not shown).

Differential analyte expression in CD patients in remission and with active disease

The same 11 analytes were measured during the follow-up of 39 CD patients of the consecutive cohort (see table 2 for patient characteristics). Patients in remission at follow-up blood collection (n=29) had a median follow-up duration of 35 months (IQR 13-55), which was not significantly different from patients with an exacerbation (n=10, median follow-up of 32 months (11-48), *P*=0.58).

Compared with baseline, many analyte levels dropped during remission (see table 3 and figure 3): sTNF-R1 (*P*=0.001), sTNF-R2 (*P*=0.001), sIL-2R (*P*=0.001), MMP-1 (*P*=0.001), S100A8 (*P*=0.002), CXCL-13 (*P*=0.012), CCL-19 (*P*=0.021), sIL-6R (*P*=0.025) and CCL-5 (*P*=0.037). During remission there was no significant difference from the levels of healthy controls, except for sTNF-R1, which was 6.7-fold lower in remission patients than in controls (*P*=0.015, table 3).

In patients with an exacerbation at the time of follow-up blood collection, there was an overall decrease of analyte levels compared with baseline (table 3 and figure 3), but this was only statistically significant for sTNF-R1 (*P*=0.007). In the patients with an exacerbation, the following followed-up analyte levels were higher than in healthy controls: sIL-2R (*P*=0.003), CCL-19 (*P*=0.007), CXCL-13 (*P*=0.028) and MMP-1 (*P*=0.031). Levels of sIL2-R, CCL-19 and CXCL-13, as well as sTNF-R1, were also significantly higher in patients with an exacerbation than in patients in remission (sIL-2R (*P*=0.012), CCL-19 (*P*=0.001), CXCL-13 (*P*=0.002), sTNF-R1 (*P*=0.003)). We were unable to determine the effect of treatment modality on analyte levels during follow-up, due to fragmentation of patient groups.

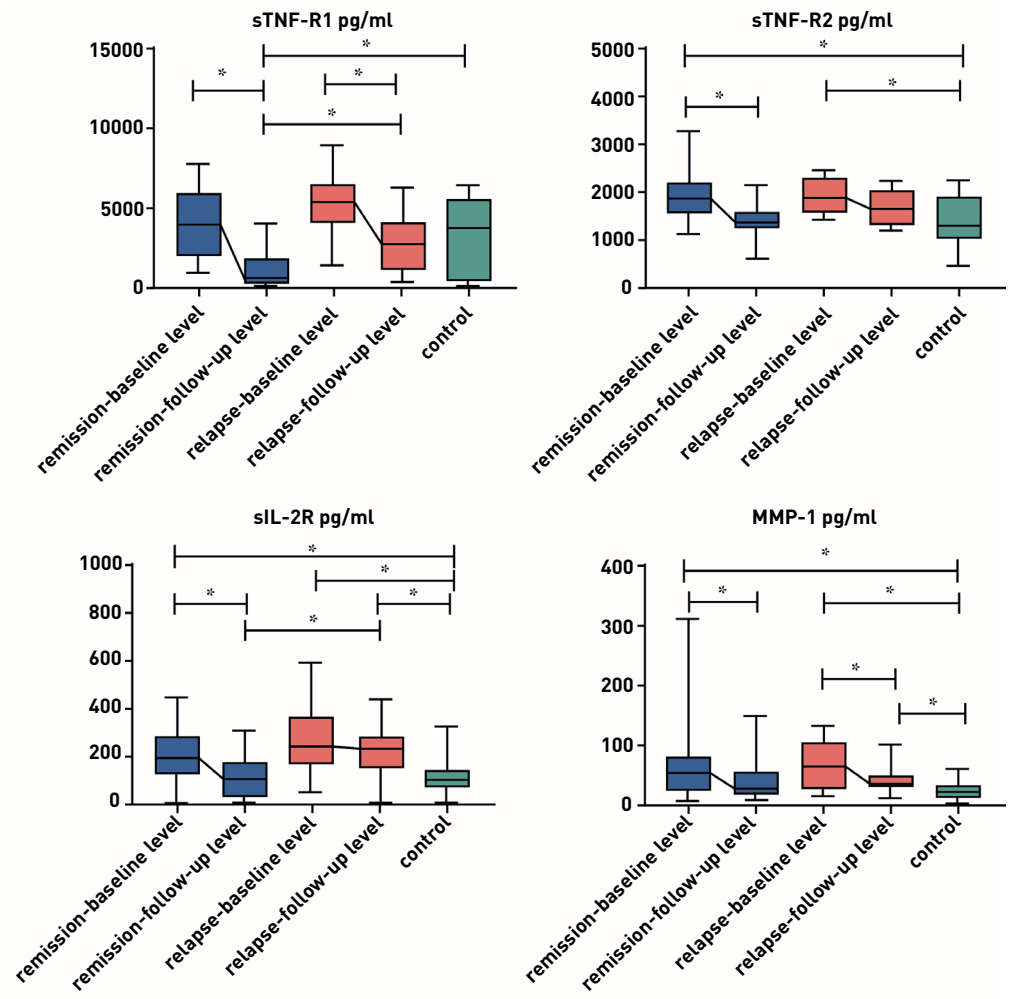

Figure 3. Crohn's disease patient's serum analyte levels measured in pg/ml and transformed to ng/ml for MMP-1, shown as baseline- and follow-up levels.

In blue levels of the group that was in remission at follow-up blood collection (n=29) and in red the group with an exacerbation at follow-up blood collection (n=10). Healthy controls are shown in green. *P-value < 0.05. MMP = matrix metalloproteinase.

Determination of optimal analyte thresholds

Based on these results we aimed to determine the optimal thresholds of baseline and follow-up analytes to discriminate different groups of patients. For each analyte, we constructed ROC curves and used Youden´s index to define the optimal threshold.

To discriminate CD and controls, baseline sTNF-R2 showed a good sensitivity (91%) but a relatively low specificity (60%), whereas sIL-2R and MMP-1 had good specificity (95%) but

relatively low sensitivity (59 and 50% respectively) (supplementary figure 1 and supplementary table 4). SIL-2R was the best single marker with an area under the curve (AUC) of 0.774 (supplementary figure 1). We reached an even higher AUC by combining the three markers (0.845) corresponding to a sensitivity of 62% and a specificity of 95% (supplementary figure 1 and supplementary table 4).

Baseline sTNF-R2 was the best analyte to discriminate initial steroid non-responders from responders with an optimal threshold at 2371.58 pg/ml corresponding to an AUC of 0.845, a sensitivity of 89% and a specificity of 86% (figure 4 and supplementary table 5).

Baseline VCAM and sTNF-R2 had good sensitivity and acceptable specificity to discriminate between CD patients with future relapsing disease and CD patients with long-lasting remission. The most accurate single marker was sTNF-R2 with an AUC of 0.693 and when combining both analytes the AUC increased to 0.738 corresponding to a sensitivity of 50%, a specificity of 97% and both positive and negative predictive values higher than 70% (supplementary figure 2 and supplementary table 6).

Follow-up analytes sIL-2R, CCL-19, CXCL-13 and sTNF-R1 were all able to discriminate between CD patients with a current exacerbation and patients in remission with sensitivity and specificity above 70% (figure 5 and table 4). The combination of these markers gave a very good discrimination between the two groups with an AUC of 0.914 and corresponding sensitivity of 80%, a specificity of 93%, positive and negative predictive values of 80 and 93% respectively.

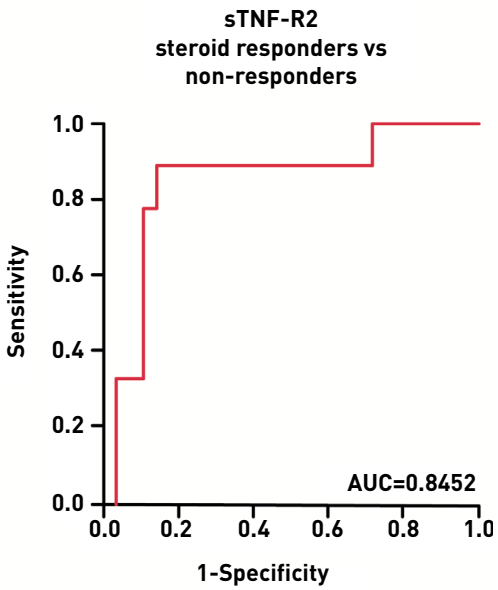

Figure 4. Receiver operating characteristic curve of baseline sTNF-R2 in Crohn's disease patients to differentiate steroid responders from non-responders. AUC = area under the curve.

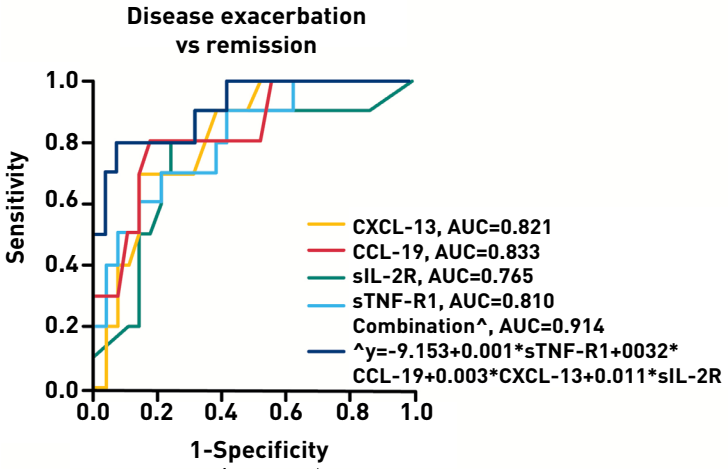

Figure 5. Receiver operating characteristic (ROC) curves of follow-up serum analytes (alone and combined) in Crohn's disease patients, for differentiation between disease exacerbation and remission at that moment.

AUC = area under the curve. Combination = CXCL-13 + CCL-19 + sIL-2R + sTNF-R1.

Table 4. Thresholds of follow-up serum analytes in Crohn's disease patients, to differentiate disease exacerbation from remission

	Threshold in pg/ml	Youden's index	Area under the curve	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
sTNF-R1	2035	0.493	0.810	70%	79%	54%	88%
CCL-19	135	0.628	0.833	80%	83%	62%	92%
CXCL-13	44	0.562	0.821	70%	86%	64%	89%
sIL-2R	128	0.590	0.765	90%	69%	50%	95%
Combined^	0.3912807	0.731	0.914	80%	93%	80%	93%

^y=-9.153 + 0.001*sTNF-R1 + 0.032*CCL-19 + 0.003*CXCL-13 + 0.011*sIL-2R

DISCUSSION

In the present study, several cytokines, chemokines, soluble receptors, growth factors, and other mediators were analysed in the sera of newly diagnosed, untreated CD patients. At time of primary diagnosis, these patients had significant higher levels of sTNF-R2, sIL-2R, and MMP-1 compared with healthy controls. Higher sIL-2R was an independent factor associated with ileocolonic disease over ileal disease. Furthermore, sTNF-R2 and sIL-2R were independently associated with endoscopic disease severity. Serial measurements revealed that sTNF-R1, sTNF-R2, sIL-2R, and MMP-1 were candidate markers of disease activity, as they all dropped significant from baseline levels during remission and were high during exacerbation. Initial non-response on prednisone therapy was associated with higher baseline levels of sTNF-R2. Furthermore, higher baseline values of sTNF-R2 and VCAM were associated with an indolent disease course without relapses, whereas lower levels were associated with relapsing disease. Remarkably, almost all pro-inflammatory cytokines, except for IL-6 and IL-18, were undetectable at baseline in most of the CD patients in the pilot cohort. This includes members of the IL-1 superfamily (IL-1a, IL-1b, IL-33), those responsible for a Th17 response (IL-12, IL-17 and IL-23), a Th1 response (IFN-g) and a Th2 response (IL-13 and IL-33), all implicated in the pathogenesis of IBD [17]. Particularly striking is the fact that TNF-a is undetectable in serum from 16 out of 20 patients. Reported series on serum TNF-a are conflicting, differing from raised levels [22] to normal [23] or undetectable [24]. As raised TNF-a concentrations have been found in the inflamed mucosa of IBD patients [25], an undetectable serum TNF-a concentration suggests a sequestration of pro-inflammatory cytokines from the systemic circulation towards the inflamed gut mucosa or increased binding to soluble TNF receptors (sTNFRs). This may put forward an explanation of the ineffectiveness of Etanercept- an engineered human sTNF-R2, preferentially neutralizing soluble TNF-a [26], as opposed to the beneficial therapeutic effects of Infliximab and Adalimumab, which bind both soluble and membrane-bound TNF-a [27].

In the consecutive cohort of patients, baseline levels could not predict the development of severe disease complications like perianal disease or stricturing and penetrating disease. However, we still cannot rule out the value of baseline serum analytes in predicting occurrence of complications in CD, since longer follow-up duration (over 10 years) is needed to conclude on this topic. Furthermore, in accordance with the numbers in our study, it is known that 20-30% of patients have already developed complications at time of diagnosis [1,2]. We did find an association of lower baseline serum levels of sTNF-R2 and VCAM with relapsing disease behaviour, whereas higher levels raised the probability of more quiescent disease after primary treatment. The combination of both markers demonstrated the best predictive value (AUC 0.738). VCAM is expressed by activated endothelium and regulates lymphocyte, monocyte, eosinophil and basophil adhesion, promoting their recruitment into the inflamed mucosa. In line with our results, positive serum VCAM levels were previously shown to be predictive of sustained clinical remission after infliximab cessation [28]. The authors hypothesized that

this soluble adhesion molecule may prevent adherence of inflammatory cells to the vascular endothelium by competition, thereby diminishing gut inflammation.

Our serial analyte measurement in patients (a group in remission and a group during exacerbation at blood collection) ensured an analysis of the evolution of these different analytes during the course of disease. The fact that most serum analyte levels (10/11) dropped in CD patients in remission, is an important observation as this implies direct association of these serological markers with mucosal disease activity in CD. Our results therefore demonstrate that the value of serum analytes in CD patients should be analysed and interpreted in combination with disease activity. Further research with a different design needs to be initiated to study the effect of different treatment modalities on serum analytes in larger (sub)groups.

Remarkably, whereas most followed-up levels in remission were comparable to those from healthy controls, the followed-up level of sTNF-R1 was almost 7-fold lower in patients in remission compared with healthy controls. In patients with an exacerbation at follow-up, sTNF-R1 levels also dropped compared with their baseline levels, however they were five times higher than in patients during remission and comparable to healthy controls. This finding on sTNF-R1 gives an insight into the initial immune response in CD patients.

The evolution over time hints at a possible role for serial measurement of sTNF-R1 to monitor disease activity. STNFRs are able to neutralise TNF, preventing its interaction with membrane-bound receptors and therefore functioning as physiological attenuators [29]. Membrane bound TNF-R1 is ubiquitously expressed and binds soluble and transmembrane TNF-a, and its function is primarily pathogenic, inducing inflammation, tissue degeneration and apoptosis [30]. On the other hand, membrane bound TNF-R2 preferentially binds transmembrane TNF-a and its function is primarily homeostatic, inducing tissue regeneration, cell proliferation and cell survival.

Administration of TNF to humans was shown to induce higher sTNF-R1 and sTNF-R2, probably by a shedding mechanism [31,32]. In our study, higher levels of sTNF-R2, but not sTNF-R1 were found in serum of CD patients compared to controls at baseline. As shedding of sTNF-R2 belongs to the armoury of regulatory T lymphocytes to inhibit TNF-a mediated inflammation, these higher baseline levels and its decline during disease remission might portray the body's attempt to inhibit the TNF-a proinflammatory downstream effects [33-35]. The fact that higher sTNF-R2 baseline values were associated with prednisone non-response and early step-up treatment could represent another disease mechanism in these patients. Elevated baseline sTNF-R2 might be associated with high mucosal TNF levels and a defective TNF-TNFR2 binding at the site of active inflammation, leading to increased shedding of sTNF-R2 in the circulation.

This group of patients might benefit more from anti-TNF treatment early in the course of disease, than might patients with low baseline sTNF-R2 levels. In two other studies in treated CD patients with a disease duration over nine years, elevated sTNFRs were found in patients with clinically active disease [24,36]. One study found increased sTNFR levels in all CD patients,

both during remission and active disease [36] whereas the other study described significantly lower levels of both sTNFRs in patients in remission. They also showed that sTNF-R2 levels decreased after infliximab therapy whereas it did not affect sTNF-R1 [24]. In rheumatoid arthritis and sarcoidosis patients, similar results were obtained where higher baseline levels of sTNF-R2 correlated with (prolonged) therapeutic response to anti-TNF treatment [34,37]. In the present study sTNF-R2 showed an accuracy of AUC = 0.845 in predicting steroid response in early CD patients. It would be interesting to further investigate whether sTNF-R2 could act as a marker in determining which CD patients will benefit most from steroids or anti-TNF treatment.

Next to the sTNFRs, sIL-2R and MMP-1 also emerged as potential markers of disease activity. Both were elevated at baseline compared with healthy controls, and decreased in patients in remission at follow-up and higher levels were found at follow-up in patients during exacerbation compared with healthy controls. Furthermore, sIL-2R was also independently associated with ileocolonic disease and a severe endoscopic score. The membrane-bound receptor for interleukin-2 is expressed among many leukocytes such as activated T cells and regulatory T cells, natural killer cells, activated B cells, monocytes, and eosinophils [38]. Proteolytic cleavage seems to be responsible for the production of circulating sIL-2R. In line with our results, sIL-2R levels were previously found to be elevated in CD patients (under treatment) compared with controls, and levels correlated with disease activity and treatment response [39-43]. Despite its potential as biomarker, demonstrated in several studies, sIL-2R has not yet reached clinical practice. In sarcoidosis, sIL-2R has been proposed as marker of disease activity [44]. Increased levels have also been observed in diseases like rheumatoid arthritis and systemic lupus erythematosus [45,46]. Therefore, sIL-2R cannot be used as diagnostic tool but might be interesting as a marker of disease activity in CD.

Matrix metalloproteinases (MMPs) are enzymes with an important role in tissue remodelling, as they are capable of degrading extracellular matrix proteins. MMP-1 breaks down interstitial collagens and has been suggested to have an anti-fibrotic role, as its overexpression attenuated liver- and cardiac fibrosis in animal models [47,48]. Previously, MMP-1 plasma concentrations were found to be elevated in patients with active ulcerative colitis compared with controls [49,50]. However, to our knowledge, MMP-1 serum levels have not been evaluated in CD patients. MMP-1 expression was reported to be increased in biopsy specimens of inflamed colonic mucosa of IBD patients and showed a good correlation with the histological degree of inflammation [51,52]. In accordance with ulcerative colitis we find higher baseline levels of MMP-1 in active CD compared with controls. We extended on this finding with its decrease in serial measurements in patients during remission, whereas levels during exacerbation were higher than in healthy controls. Therefore, we believe MMP-1 might be a useful marker of disease activity. MMP-9 seems to be an important marker in CD [53]. Unfortunately, as MMP-9 levels in our study were all extrapolated above standard range (both in patients and in healthy controls), these values were not reliable for further analysis. However, we do believe that it would be worthwhile to further investigate this marker using higher dilutions.

CCL-19, CXCL-13, sTNF-R1, and sIL-2R could differentiate between remission and exacerbation at follow-up blood collection. CCL-19 was the most accurate single marker (AUC = 0.833), but the best accuracy was reached by a combination of these four analytes (AUC = 0.914). Further research is needed to elucidate if the combination of these markers can be used to identify patients with a disease exacerbation, without the need for endoscopy or faecal calprotectin examination. Chemokines CXCL-13 and CCL-19 are known to be required for the development and maintenance of secondary and tertiary lymphoid organs, as well as the entry of lymphocytes and dendritic cells into these lymphoid organs [54,55]. Circulating levels possibly reflect the expression in the inflamed gut. Serum and mucosal CXCL-13 was found to be elevated in IBD patients compared with controls [56-58]. The differentiating potential of elevated serum levels of CCL-19 and CXCL-13 between exacerbation and remission might be explained by the formation and resolution (respectively) of tertiary lymphoid organs in the inflamed gut [59]. Previous studies on mediators of inflammation in CD patients have assessed individual analytes or combined a limited set of analytes in serum or intestinal mucosa. In these studies, heterogeneous patient cohorts with divergent disease durations and IBD phenotypes were included, and almost invariably patients were receiving immunomodulatory treatment. A recent literature overview identified 89 research papers on cytokines and chemokines in IBD patients, with a median sample size of 31 CD patients mainly receiving pharmacological therapy [18]. Results of these studies are therefore inconclusive, as analyte levels are associated with disease activity and potentially also with treatment modality. Our serial results showed that patients in remission and patients in relapse displayed different evolution of serum analyte profiles and therefore these results should be analysed and interpreted separately.

We identified several limitations and possible explanations for the difference in results between the pilot cohort and the consecutive cohort. Due to the exploratory nature of the pilot group, no adjustments were made to the *P*-value for multiple comparisons. Furthermore, as the sample size was small we could not perform multivariate analysis. Due to the disease heterogeneity in CD we were limited by relatively small groups of different disease phenotypes. The follow-up duration of patients was probably too short to determine the true value of these baseline analyte levels in predicting future disease course with respect to the development of complications.

The strength of our study is that we analysed a full spectrum of analytes in two well-characterised cohorts of CD patients at the time of diagnosis, all with endoscopically confirmed inflammation. Our baseline results are not biased by the influence of therapy, as all patients were untreated. Compared with other papers on this topic, we had access to a relatively large group of CD patients and performed serial measurements during follow-up. Our analysis was based on released proteins and not on mRNA levels which can be hampered by regulatory events that may inhibit the translation of mRNA to protein. Here, we extended on the proposed immunological basis for CD heterogeneity, with several candidate serum analytes that might reflect the underlying complex immune processes in CD and seem to be logical and promising candidates as non-invasive biomarkers to predict disease activity and course.

CONCLUSION

There is a very small window of opportunity for the analysis of early inflammatory processes in CD patients, as therapy that influences the immune system is usually initiated soon after diagnosis. The serial measurements in patients at diagnosis and during follow-up identified sTNF-R1, sTNF-R2, sIL-2R and MMP-1 as potential markers of disease activity. Furthermore, sTNF-R2 and VCAM were associated with prednisone response and disease course. These candidate markers are easily accessible and implementable in daily practice and therefore warrant further investigation.

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SUPPLEMENTARY TABLES AND FIGURES

Supplementary table 1. Analyte serum concentrations and clinical phenotype at diagnosis of CD patients.

MONTREAL CLASSIFICATION	ANALYTE Median (Min-Max)						
	sTNF-R1 pg/ml	sTNF-R2 pg/ml	sIL-2R pg/ml	S100A8 ng/ml*	VCAM ng/ml*	IL-37 pg/ml	MMP-9 ng/ml*
L1 (n=4)	2803 (1414-6066)	1760 (1692-2006)	248 (149-469)	11 (5-26)			
L2 (n=4)	7744 (4451-12559)	2932 (2082-4327)	1207 (536-2241)	26 (1128)			
L3 (n=12)	4324 (2879-7936)	2184 (1531-4202)	571 (220-1118)	6 (3-21)			
+P (n=5)					972 (664-1751)	275 (10-1067)	9700 (6560-11500)
Not +P (n=15)					1514 (1086-3000)	10 (10-374)	11700 (3490-12000)
P-value	L1 vs. L2 0.026	L1 vs. L2 0.026	L1 vs. L2 0.008	L3 vs. L2 0.022	0.025	0.033	0.025

* Please note that this unit (ng/ml) differs from the other analyte units in this table (pg/ml)

Supplementary table 2. Analyte serum concentrations at follow-up of CD patients.

	Steroid non-responder (n=3, 18%)	Steroid responder (n=14, 82%)	Maintenance treatment at follow-up (n=16, 80%)	No maintenance treatment at follow-up (n=4, 20%)	Anti-TNF medication (n=10, 50%)	No Anti-TNF medication (n=10, 50%)	Endoscopic proven relapse (n=9, 45%)	No endoscopic proven relapse (n=11, 55%)	*P*-value
Follow-up[a]	40.0 (15-54)*	48.5 (10-63)*	47.5 (16-60)^	48.5 (10-63)^	57 (40-63)~	40.5 (10-60)~	54 (39-63)#	43 (10-60)#	*0.362 ^0.892 ~0.005 #0.056
CCL-19 pg/ml[b]	132 (110-139)	62 (20-108)							0.003
sIL-2R pg/ml[b]	1118* (711-2241)	453* (149-969)	588 (291-2241)^	185 (75-790)^					*0.032 ^0.050
TNF-R1 pg/ml[b]			5806 (2093-12559)	3196 (1414-4335)					0.039
CCL-5 pg/ml[b]			147 (70-236)*	61 (42-122)*			190 (85-236)^	86 (42-162)^	*0.016 ^0.007
SIL-6R pg/ml[b]			23 (17-29)*	31 (23-41)*	23 (17-27)^	27 (21-41)^			*0.022 ^0.009
CXCL-13 pg/ml[b]					51 (34-198)	26 (9-799)			0.035
MMP-1 ng/ml[b]							83 (32-417)*	46 (9-105)*	*0.046

[a]Follow-up period in months, median (min-max)

[b]Analytes in pg/ml, except for CCL-5, sIL-6R and MMP-1 (ng/ml), median (min-max)

*^~# symbols are used to indicate the P-value corresponding to different clinical comparisons of the same analyte

Supplementary table 3. Number of patients with undetectable serum analytes in the Crohn's disease patients and healthy controls

Serum analytes	CD patients pilot cohort (n=20)	CD patients at baseline (n=66)	CD patients at follow-up (n=39)	Healthy controls (n=20)
IL-1α	13	-	-	-
IL-1β	17	-	-	-
IL-1RA	2	-	-	-
IL-6	2	-	-	-
IL-10	12	-	-	-
IL-12	11	-	-	-
IL-13	10	-	-	-
IL-17	10	-	-	-
IL-18	0	-	-	-
IL-21	0	-	-	-
IL-23	10	-	-	-
IL-33	9	-	-	-
IL-37	12	53	32	15
TNF-α	16	-	-	-
IFN-γ	14	-	-	-
CCL-2	0	-	-	-
CCL-3	0	-	-	-
CCL-4	0	-	-	-
CCL-5	0	0	0	0
CCL-19	0	0	0	0
CXCL-8	0	-	-	-
CXCL-10	0	-	-	-
CXCL-13	0	0	0	0
GM-CSF	15	-	-	-
EGF	0	-	-	-
VEGF	0	-	-	-
sICAM	0	-	-	-
sVCAM	0	0	0	0
sTNF-R1	0	0	0	0
sTNF-R2	0	0	0	0
sIL-2R	1	2	5	2
sIL-6R	0	0	0	0
MMP-1	0	0	0	0
MMP-3	0	-	-	-
MMP-9	0	0	0	0
S100A8	0	0	0	0

- = not measured in the consecutive cohort or healthy controls

Supplementary table 4. Thresholds of baseline serum analytes in Crohn's disease (CD) patients to differentiate CD from HC.

	Threshold	Youden's index	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
sTNF-R2	1403	0.509	91%	60%	88%	67%
sIL-2R	172	0.541	59%	95%	97%	41%
MMP-1	51686	0.450	50%	95%	97%	37%
Combined	0.8294077	0.571	62%	95%	98%	43%

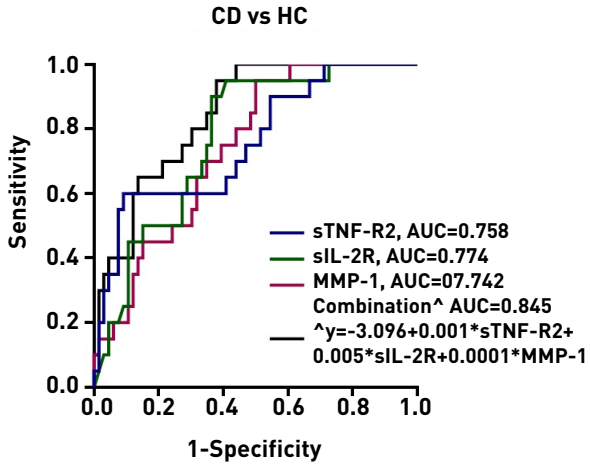
Supplementary table 5. Threshold of baseline sTNF-R2 in Crohn's disease patients to differentiate steroid responders from non-responders.

	Threshold in pg/ml	Youden's index	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
sTNF-R2	2372	0.746	89%	86%	67%	96%

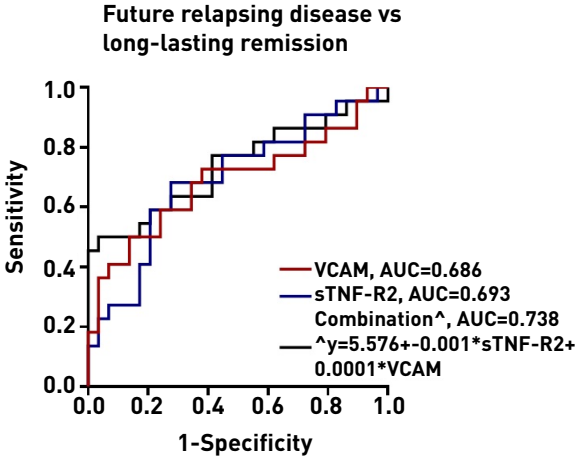
Supplementary table 6. Thresholds of baseline serum analytes in Crohn's disease patients to differentiate future relapsing disease from long-lasting remission.

	Threshold in pg/ml	Youden's index	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
sTNF-R2	1996	0.406	72	68	75%	65%
VCAM	1876350	0.362	86	50	69%	73%
Combined^	0.4426898	0.466	50	97	92%	72%

^ y = 5.576 + -0.001*sTNF-R2 + 0.0001*VCAM



Supplementary figure 1. Receiver operating characteristic (ROC) curves of baseline serum analytes (alone and combined) for differentiation between Crohn's disease (CD) and healthy controls (HC). AUC = area under the curve.



Supplementary figure 2. Receiver operating characteristic (ROC) curves of baseline serum analytes (alone and combined) to differentiate future relapsing disease from long-lasting remission. AUC = area under the curve.

8

Discussion and summary
Nederlandse samenvatting
Abbreviations

DISCUSSION AND SUMMARY

The driving immune factors involved in the early stages of inflammatory bowel disease (IBD) are still poorly understood, as most studies investigate heterogeneous patient cohorts, including both active and inactive disease, with variable disease duration, and under various types of anti-inflammatory treatment. However, on top of the list of key research priorities regarding optimization of IBD treatment strategy is selection of the right patient group and phase of disease, and identification of markers for patient stratification regarding disease course [1]. In this thesis we investigated the dysregulated immune response in IBD patients, with a focus on the T lymphocyte compartment, to define immunological biomarkers for disease development and prognosis. We studied patients at diagnosis, and during their follow-up, both at local (intestinal mucosa) and systemic (serum) level. We related the immune characteristics to disease activity and course of disease. Differences in mucosal T lymphocyte subsets or serum analytes at diagnosis and in the course of IBD might have predictive value for disease course or response to different therapeutic agents. However, before they can be translated to clinical practice, the first steps in this process are identifying potential biomarkers and analysing their behaviour in IBD patients over time.

Intestinal T lymphocytes

In **chapter 2-5** we focused on T lymphocyte subsets in the intestinal mucosa of IBD patients at diagnosis and during follow-up. Our model of investigation consisted of biopsied specimens of the gut mucosa from newly diagnosed, untreated IBD patients at the time of their first presentation and during their follow-up as well as biopsied specimens from healthy controls. Several T lymphocyte populations were analysed by flow cytometric and immunohistochemical analysis. Two comprehensive literature reviews were performed to investigate the current knowledge on T lymphocyte subsets.

T lymphocyte activation, maturation and tertiary lymphoid organs

In the literature review in **chapter 2**, we discussed the pathways of T lymphocyte activation, maturation and migration to the gut mucosa in IBD, especially of the naïve T lymphocytes (T_N), which are normally thought to be excluded from inflamed tissues. Classically, these T_N are believed to recirculate between lymph, blood and secondary lymphoid organs (SLOs), e.g. lymph nodes (LN), gut associated lymphoid tissue (GALT); found at predefined sites and developed before birth. Tertiary lymphoid organs (TLOs) are ectopic accumulations of lymphoid cells which develop postnatally in response to inflammation or inflammatory cues in patients with autoimmune disease or cancer [2]. In LN, T lymphocytes and DCs are directed to the paracortical region by CCL-19 and CCL-21, and B cells are directed to follicles by CXCL-13. These chemokines are also expressed in TLOs and can facilitate the TLO entry of circulating CCR-7^+ T_N and T_{CM} lymphocytes. We presented the growing signals of the presence of TLO

formation in the inflamed mucosa and submucosa of IBD patients, potentially facilitating the homing of T_N and T_{CM} lymphocytes to the gut through the high endothelial venules (HEVs) present in TLOs. This could allow T lymphocytes to differentiate into effector cells, after contact with their cognate antigen, independent of SLOs. This pathway could explain the observed presence of T_N and T_{CM} lymphocytes in the inflamed intestine. Furthermore, it is conceivable that targeted therapy against TLOs could restore immunologic homeostasis in IBD patients, as they are thought to exacerbate inflammation in autoimmune diseases, whereas in cancer, their presence predicts a favorable outcome, suggesting it to be a site of antigen activation and anti-tumor immunity [2].

We explored TLO formation in the gut and the presence of T lymphocytes at different stages of maturation in the inflamed intestine of IBD patients in **chapter 3**. HEVs were studied by immunohistochemistry with staining of MECA-79, and lymphocytes subsets were identified by flowcytometric immunophenotyping. We described our finding of a higher number of extrafollicular HEVs in the inflamed intestine of newly diagnosed IBD patients compared to the uninflamed intestine of healthy controls (HC). A high extrafollicular HEV density in the gut (HEV^{high}) was associated with increased gut infiltration of T_N and T_{CM} compared to HEV^{low} patients. HEV^{high} patients also displayed a higher number of colonic follicles in their biopsied specimens compared to HEV^{low} patients, as well as upregulated serum chemokines associated with TLO formation (CXCL-13 and CCL-19). It was our interpretation that the extrafollicular HEVs represent an early phase of TLO formation in the inflamed mucosa, as these vessels were absent in healthy controls.
Future research should elucidate the true function of TLOs in IBD pathogenesis and if $HEV^{high/low}$ distinction can serve as biomarker in IBD patients. Analyzing HEVs and TLOs during disease course is necessary to elucidate whether they are present after treatment and in disease remission. It would also be an interesting topic for further research to study if HEV^{low} patients benefit more from anti-α4b7 therapy than HEV^{high} patients, as HEV^{low} patients have higher percentages of gut T_{EM} lymphocytes, generally thought to be $\alpha4\beta7^+$. HEV and TLO formation could also be tested as a new treatment strategy; a candidate target is TNFR signaling, as it has been shown to induce ectopic HEVs whereas it is not required for maintenance of HEVs in SLOs [3].

In **chapter 4,** we identified the intestinal T lymphocyte infiltrate in IBD patients with active endoscopic disease at diagnosis and during follow-up. The T lymphocyte infiltrate was studied by flow cytometry identifying several lymphocyte subpopulations in the biopsied specimens. The infiltrate during active endoscopic disease was composed of increased percentages of $CD4^+$ T lymphocytes, Tregs and T_{CM} lymphocyte, with lower percentages $CD8^+$ T lymphocytes and $CD103^+$ T lymphocytes compared to HC, and endoscopic inactive IBD. When patients entered endoscopic remission, lymphocyte subset composition of the gut recovered to

percentages comparable with HC, irrespective of treatment strategy. Baseline percentages of T lymphocyte subsets were associated with disease outcome. In patients with ulcerative colitis (UC), lower baseline percentages of $CD3^+$ lymphocytes was associated with a milder disease course, without the need of step-up to an immunomodulator. In CD patients, higher baseline percentages of $CD4^+$ lymphocytes and higher percentages of Tregs were associated with a more complicated disease course; the development of strictures or penetrating disease. This might be explained by increased invasion of proinflammatory $CD4^+$ lymphocytes accompanied by a subsequent, but insufficient, Treg expansion.

Future studies in another IBD patient cohort should confirm the predictive value of intestinal percentages of $CD4^+$ lymphocytes and/or percentages of Tregs in Crohn's disease (CD) patients, as well as percentages of $CD3^+$ lymphocytes in UC patients, and cut-off values should be established. Flow cytometric marker panels performed on (human) tissues are almost solely based on, and extrapolated from, blood-based studies. Therefore, it would be interesting to investigate other markers in the intestinal gut mucosa of IBD patients and healthy controls regarding T lymphocyte maturation (CCR-7, CD62L, CD45RO, CD28, and CD31) and T lymphocyte proliferation (CD69, KI-67) to define the optimal gut mucosal marker panel. Functional studies in newly diagnosed IBD patients, for example investigation of local cytokine production of different intestinal T lymphocyte subsets, would enhance our understanding on the specific role of different T lymphocyte subsets in the early inflammatory processes.

αEβ7 integrin

Anti-integrin therapy targeting T lymphocyte trafficking to the gut is emerging as a new treatment option for IBD patients. The transmembrane integrin αEβ7 (CD103) on T lymphocytes is thought to contribute to T lymphocyte retention within the gut, therefore blockade by anti-β7 treatment, potentially disrupts this retention (phase three trials in IBD patients ongoing). Furthermore, by intervening with the α4β7/MAdCAM-1 interaction, anti-β7 treatment should also block the gut homing of $\alpha4\beta7^+$ lymphocytes. **Chapter 5** encompasses a critical overview on the immune cells expressing integrin αEβ7, with a focus on their homeostatic roles (in health) and potential pathogenic roles (in IBD). The letter displayed in **chapter 5a** challenges the proposed pathogenic role of $\alpha E\beta7^+$ T lymphocytes in IBD patients, as our group showed that newly diagnosed, untreated IBD patients have statistically significant lower percentages of $\alpha E\beta7^+$ T lymphocytes in their inflamed intestinal biopsy specimen compared to HC [4]. Furthermore, we demonstrated that patients who entered endoscopic remission had increased percentages of intestinal $\alpha E\beta7^+$ T lymphocytes, which became comparable to the $\alpha E\beta7^+$ T lymphocyte percentages of HCs. Therefore, $\alpha E\beta7^+$ T lymphocytes might even be necessary to achieve/maintain remission in IBD patients.

A detailed review of the topic was presented in **chapter 5b**. Next to its expression on pro-inflammatory T lymphocytes, αEβ7 is also expressed on other (regulatory) immune cell subsets (e.g. Tregs, DCs), and in other organs than the gut (e.g. the brain, lungs, liver, kidneys). This

might lead to unwanted adverse events in IBD patients treated with anti-β7 therapy, targeting both α4β7 and αEβ7 immune cells. The development of anti-β7 treatment was prompted by the idea of increased $\alpha E\beta 7^+$ T lymphocytes in the inflamed intestine of IBD patients, implying a pathogenic role for these cells. However, this has not yet been demonstrated. On the opposite, lower percentages of $\alpha E\beta 7^+$ T lymphocytes are found in IBD patients compared to HC. Further research on this topic should identify subsets and functionality of the $\alpha E\beta 7^+$ intestinal T lymphocyte subsets in IBD patients. This could be done by functional assays, also studying TGF-beta and retinoic acid. Subset analysis should be done with other markers like CD69, CD4/CD8 determination, markers of T lymphocyte maturation, but also studying Tregs and DCs along the intestine. This could define specific subgroups of patients that might benefit from anti-β7 treatment.

Serum biomarkers

Readily available, non-invasive, accurate markers of disease activity that can predict future disease course or response to treatment would substantially contribute to early patient stratification in IBD. For this thesis we investigated several candidate serum markers in newly diagnosed, untreated IBD patients (**chapter 6 and 7**). Our model of investigation consisted of the serum IBD patients at diagnosis and during their follow-up, as well as serum from HCs. Serological antibodies were studied by enzyme-linked immunosorbent assays and indirect immunofluorescence. Cytokines, chemokines, growth factors, soluble receptors and other mediators were studies by multiplex immunoassays.

Serological antibodies

Despite intensive investigation of serological antibodies in adult IBD patients, these studies were single time point studies after disease complications had already occurred. In **chapter 6** we assessed serum antibodies in newly diagnosed IBD patients at diagnosis, and during their follow-up. The combination of pANCA and ASCA enabled good discrimination between CD and UC patients. No associations were found with antibody presence at diagnosis and disease outcomes. Overall, antibody prevalence in our real-world IBD study was relatively low compared to the prevalence in previous, referral-based, studies [5,6]. Antibody presence at baseline showed good correlation with serial measurement, but its concentrations did fluctuate over time, sometimes related to thiopurine use and anti-TNF treatment. Therefore, it would be interesting to study these antibodies in homogenous patient cohorts, before and after starting the same therapeutic agent (for example infliximab) to reveal if antibody status and/or magnitude changes with drug treatment and with treatment response. The appearance and disappearance of pANCA in UC over time correlated with disease activity, and might be used in disease monitoring as it also showed strong correlation with calprotectin values. Future investigations with longitudinal investigation of pANCA in UC patients over time during several exacerbations should reveal if it can be used as a monitoring marker (in a subgroup of patients).

If new serological antibodies are tested in IBD patients, we prompt their analysis in a real world IBD population, preferably newly diagnosed patients with longitudinal serum collections.

Candidate serum markers

In **chapter 7** we identified candidate serum markers by multiplex immunoassays in untreated CD patients at diagnosis and studied these markers during follow-up. Serum levels of sTNF-R2, sIL-2R and MMP-1 were higher in CD patients than in HCs, and dropped significantly from baseline levels during disease remission, while they rose during disease exacerbation. Almost all pro-inflammatory cytokines, implicated in the pathogenesis of IBD, were undetectable in our CD patients. This could suggest a sequestration of these cytokines towards the inflamed gut mucosa or increased binding to soluble receptors. Serial measurements identified a statistically significant drop of most analyte levels from baseline levels, only in patients in remission at follow-up. This suggests an association between serum marker levels and mucosal disease activity. At follow-up, the combination of CCL-19, CXCL-13, sTNF-R1, and sIL-2R could identify patients with disease exacerbation from patients in remission with good accuracy. The combination of these markers might hold the potential to identify disease exacerbation in patients without the need for (invasive) endoscopy.

Lower baseline sTNF-R2 and VCAM serum levels were associated with relapsing disease behaviour and higher baseline sTNF-R2 levels were associated with initial non-response to oral steroid therapy.

Future research should reveal the predictive value of the mentioned serum analytes in IBD patients. In line with our findings, sIL-2R was recently proposed as marker for mucosal healing in IBD patients [7]. As this marker is already used in clinical practice in other patient categories, for example patients with sarcoidosis, it seems to be the first designated marker to undergo further investigation in CD patients.

Studies in IBD patients on candidate markers, simultaneously analysing serum as well as intestinal supernatant could reveal the correlations between serum and gut marker levels and could give an insight on the etiological disease mechanisms.

Serum analyte investigation in newly diagnosed UC patients and during follow-up should uncover candidate markers in those patients. Furthermore, in line with our suggestion on serological antibodies, homogeneous IBD patient cohorts should be tested on serum analytes before and after starting a therapeutic agent to reveal their biomarker value, especially in predicting treatment response.

Future research

In general, the differences in immunological phenotypes we found both regarding T lymphocyte subsets as well as serum analytes, between patients with active endoscopic disease compared to healthy controls and patients in endoscopic remission, underline the necessity of distinguishing these subgroups in future studies. Even though this may sound logical, this

approach is still not common practice, as IBD patients are often compared to healthy controls, independent of the presence of intestinal inflammation.

Simultaneously to the increasing incidence of IBD, the therapeutic armamentarium is rapidly expanding, with multiple novel agents waiting to enter the market. This poses opportunities and challenges for health care workers; how to choose the most appropriate therapy for the individual patient? The current IBD literature is increasingly urging to evolve from a symptom based treatment paradigm towards a treat-to-target approach, based on objective criteria and patient-reported outcomes, to decrease the long-term burden of disease [8,9]. Biomarkers would be of great help to implement treat-to-target strategies in a personalized way.

In this thesis we identified several candidate biomarkers in IBD patients and their behaviour in patients over time. In the future, the most potent predictive biomarkers should be combined in a web-based tool to reveal a patient's risk profile, matching an individual patient with treatment and managing options, to enhance personalized medicine and disease outcomes. To reach this goal, extensive studies in homogeneous, well characterized IBD patient populations need to be performed on candidate predictive markers.

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NEDERLANDSE SAMENVATTING

Tot op heden weten we weinig over de immunologische elementen (het afweersysteem) die betrokken zijn in het vroege stadium van inflammatoire darmziekten (IBD). Dit komt doordat de meeste studies heterogene patiëntgroepen onderzoeken, met daarin zowel patiënten met actieve als patiënten met inactieve ziekte, met een wisselende ziekteduur en onder verschillende typen anti-inflammatoire medicatie. Toch staan er bovenaan de lijst met onderzoeksprioriteiten omtrent het optimaliseren van de behandelingsstrategieën bij IBD, de selectie van het juiste middel voor de meest geschikte patiëntengroep en fase van ziekte, en het opsporen van markers die kunnen voorspellen hoe het ziektebeloop van verschillende patiënten zal zijn[1]. Wij denken dat we, om deze vragen te beantwoorden, eerst meer moeten snappen over die immunologische elementen in het vroege IBD-stadium, dus aan het begin van de ziekte. In dit proefschrift onderzoeken we de verstoorde immuunrespons in IBD-patiënten, met speciale aandacht voor T-lymfocyten (afweercellen behorend tot de witte bloedcellen), in een poging tot het identificeren van immunologische markers die het ziektebeloop van patiënten kunnen voorspellen en die iets zeggen over de prognose van de patiënt. We bestudeerden patiënten met IBD op het moment van diagnose en gedurende hun ziekte, hierbij keken we op lokaal niveau (darmslijmvlies) en systemisch niveau (bloed). We relateerden immunologische karakteristieken aan ziekteactiviteit en ziektebeloop. Verschillen in typen T-lymfocyten in het slijmvlies of in analyten in het bloed bij patiënten met IBD op het moment van diagnose en gedurende de ziekte zouden voorspellende waarde kunnen hebben voor het ziektebeloop of de respons op verschillende behandelingen. Echter, de eerste stappen in dit proces zijn het identificeren van potentiële markers en het analyseren van hun gedrag in IBD-patiënten op verschillende momenten, voordat deze markers gebruikt kunnen worden in de klinische praktijk.

T-lymfocyten in de darm

In **hoofdstukken 2 tot en met 5** hebben wij ons gefocust op verschillende T-lymfocyten subsets in het darmslijmvlies van patiënten met IBD op het moment van diagnose en gedurende hun ziekte. Ons onderzoeksmodel bestond uit het onderzoeken van darmbiopten van nieuw gediagnosticeerde IBD-patiënten en op een later moment gedurende hun ziekte. Hiernaast onderzochten we ook darmbiopten van gezonde volwassenen ter controle. Verschillende typen T-lymfocyten werden geïdentificeerd met behulp van flowcytometrisch en immunohistochemisch onderzoek. Twee gedetailleerde reviews van de literatuur werden uitgevoerd om de huidige stand van zaken omtrent T-lymfocyten subsets binnen IBD op een rij te zetten.

T-lymfocyt activatie, rijping en tertiair lymfoïde organen

In de literatuurreview in **hoofdstuk 2** bediscussieerden we de manier van T-lymfocyt activatie, rijping en migratie naar het darm slijmvlies bij IBD, met de nadruk op naïeve T-lymfocyten (T_N), waarvan normaal gesproken verondersteld wordt dat deze zich niet bevinden in ontstoken weefsels. De klassieke gedachte is dat deze T_N circuleren tussen lymfevloeistof, bloed en secundaire lymfoïde organen (SLOs) zoals de lymfeklieren en de platen van Peyer; die gevonden worden op standaard plaatsen in het lichaam en ontwikkeld zijn voor de geboorte. Tertiair lymfoïde organen (TLOs) zijn ectopische ophopingen van lymfoïde cellen die zich na de geboorte ontwikkelen als reactie op ontsteking of ontstekingsprocessen in patiënten met een auto-immuunziekte of kanker [2].

In lymfeklieren worden T-lymfocyten en dendritische cellen naar de paracorticale regio gestuurd door CCL-19 en CCL-21, terwijl B-lymfocyten naar de follikels worden geleid door CXCL-13. Deze chemokines worden ook gevonden in TLOs, waar ze de toegang van CCR-7$^+$ T_N en central memory T (T_{CM}) lymfocyten kunnen faciliteren.
Wij presenteerden groeiend bewijs van de aanwezigheid van TLO vorming in het ontstoken slijmvlies van IBD-patiënten, waar ze mogelijk zorgen voor de migratie van T_N en T_{CM} lymfocyten naar de ontstoken darm via hoogendotheliale venulen (specifieke kleine bloedvaten, HEVs) aanwezig in TLOs. Dit zou er voor kunnen zorgen dat T-lymfocyten differentiëren naar effectorcellen, na contact met hun antigeen, onafhankelijk van SLOs. Deze route zou de aanwezigheid van T_N en T_{CM} lymfocyten in de ontstoken darm kunnen verklaren. Verder is het denkbaar dat therapie gericht tegen TLOs de immunologische balans in IBD-patiënten zou kunnen herstellen, waarbij de gedachte is dat TLOs opvlammingen in auto-immuunziekten kunnen bewerkstellingen. Bij kanker lijken ze juist samen te hangen met een gunstige prognose, wat suggereert dat het daarbij een plek is van antigeen activatie en anti-tumor immuniteit [2].

We onderzochten de vorming van TLOs in de darm en de aanwezigheid van T-lymfocyten in verschillende fasen van rijping in de ontstoken darmwand van IBD-patiënten in **hoofdstuk 3**. We bestudeerden HEVs met behulp van immunohistochemische kleuring van de darmbiopten met MECA-79 en de verschillende T-lymfocyten subsets werden geïdentificeerd met flowcytometrisch onderzoek. We vonden meer extra-folliculaire HEVs in de ontstoken darm van nieuw gediagnosticeerde IBD-patiënten in vergelijking met de niet-ontstoken darm van gezonde volwassenen. Het hebben van meer extra-folliculaire HEVs (een zogenaamd HEV^{hoog} profiel) was geassocieerd met een toegenomen infiltratie van T_N en T_{CM} lymfocyten in de darm in vergelijking met HEV^{laag} patiënten. HEV^{hoog} patiënten hadden ook meer follikels in hun dikke darm biopten in vergelijking met HEV^{laag} patiënten, alsmede een hogere waarde van serum chemokines die geassocieerd zijn met de vorming van TLOs (CXCL-13 en CCL-19). Het was onze interpretatie dat deze extra-folliculaire HEVs een vroege fase van TLO vorming

in de ontstoken darm weerspiegelen, omdat deze bloedvaten afwezig waren in gezonde volwassenen. Toekomstig onderzoek zal moeten ophelderen wat de functie van TLOs in de pathogenese van IBD is en of het onderscheid tussen $HEV^{hoog/laag}$ kan dienen als marker in IBD-patiënten. Het analyseren van HEVs en TLOs gedurende de ziekte is nodig om te ontdekken of ze verdwijnen tijdens behandeling, als de darm niet meer ontstoken is. Het zou interessant zijn om te onderzoeken of patiënten met een HEV^{laag} profiel meer baat hebben van behandeling met anti-α4b7 dan HEV^{hoog} patiënten, omdat HEV^{laag} patiënten hogere percentages effector memory T-lymfocyten in de darm hebben, welke voornamelijk $\alpha4\beta7^+$ lijken te zijn. De vorming van HEVs en TLOs in IBD zou ook getest kunnen worden als doelwit voor nieuwe behandelstrategieën. Een kandidaat-molecuul hiervoor is tumor necrosis factor receptor signalering, aangezien werd aangetoond dat dit de nieuwvorming van HEVs kan induceren terwijl het niet nodig is voor het behoud van HEVs in SLOs [3].

In **hoofdstuk 4** identificeerden we het infiltraat van T-lymfocyten in de darm van IBD-patiënten met actieve endoscopische ziekte op het moment van diagnose en gedurende hun ziekte. Het T-lymfocyten infiltraat werd bestudeerd met behulp van flowcytometrisch onderzoek van de darmbiopten. Gedurende actieve endoscopische ziekte bestond dit infiltraat voornamelijk uit een toegenomen percentage van $CD4^+$ T-lymfocyten, regulatoire T-lymfocyten en T_{CM} lymfocyten, met afgenomen percentages van $CD8^+$ T-lymfocyten en $CD103^+$ T-lymfocyten in vergelijking met gezonde volwassenen en endoscopisch inactieve ziekte (niet-ontstoken darm na behandeling). Als patiënten endoscopisch inactieve ziekte bereikten, herstelden alle percentages van lymfocytensubsets in de darm tot waarden die vergelijkbaar waren met gezonde volwassenen, ongeacht de behandelstrategie. Bepaalde percentages van T-lymfocyten subsets op het moment van diagnose waren geassocieerd met de uitkomst van ziekte. In patiënten met colitis ulcerosa (CU) waren lage percentages van $CD3^+$ lymfocyten geassocieerd met een milder ziektebeloop, waarbij het niet nodig was om een behandelstap omhoog te gaan (het starten van een immunomodulator). In patiënten met de ziekte van Crohn (CD) waren hogere percentages van $CD4^+$ lymfocyten en van regulatoire T-lymfocyten geassocieerd met een complexer ziektebeloop, namelijk de ontwikkeling van darmstricturen of fistelende ziekte. Dit zou mogelijk verklaard kunnen worden door een versterkte toestroom van proinflammatoire $CD4^+$ lymfocyten samen met een inadequate expansie van regulatoire T-lymfocyten.

Toekomstig onderzoek in een ander cohort IBD-patiënten zou de voorspellende waarde van de percentages van $CD4^+$ lymfocyten en/of regulatoire T-lymfocyten in CD-patiënten, alsmede de percentages $CD3^+$ lymfocyten in CU-patiënten, moeten bevestigen. Hierbij zouden ook de afkapwaarden moeten worden vastgesteld.
Flowcytometrisch onderzoek op menselijke weefsels is nagenoeg altijd gebaseerd op onderzoek in bloed. Het zou daarom interessant zijn om andere flowcytometrische markers

te onderzoeken in de darmbiopten van IBD-patiënten en gezonde controles, gericht op T-lymfocyten rijping (bijvoorbeeld CCR-7, CD62L, CD45RO, CD28, en CD31) en T-lymfocyten proliferatie (CD69, KI-67), om het optimale panel van darmmarkers te identificeren.

Functionele onderzoeken in nieuw gediagnosticeerde IBD-patiënten, bijvoorbeeld het onderzoeken van lokale cytokine productie van verschillende T-lymfocyten subsets in de darm, zou ons begrip verbeteren over de specifieke rollen van de verschillende subsets in de vroege inflammatoire fase van de ziekte.

αEβ7 integrine

Een nieuw ontwikkelde behandeloptie voor IBD-patiënten is anti-integrine therapie, welke de migratie van de T-lymfocyt naar de darm aanpakt. Er wordt gedacht dat de transmembraneuze integrine αEβ7 (CD103) op T-lymfocyten bijdraagt aan het vasthouden van T-lymfocyten in de darm, daarom zou een blokkade met een anti-β7 medicijn dit vasthouden verstoren. Op dit moment lopen de fase-3-studies van deze medicamenteuze therapie in IBD-patiënten. Daarnaast zou de anti-β7 behandeling, door het ingrijpen op de α4β7/MAdCAM-1 interactie, ook de migratie van $\alpha4\beta7^+$ lymfocyten naar de darm moeten blokkeren. **Hoofdstuk 5** omvat een overzicht van de immuuncellen die de integrine αEβ7 tot expressie brengen, met een focus op hun homeostatische rol (in gezondheid) en hun potentiële pathogene rol (in IBD). Het commentaar, te vinden in **hoofdstuk 5a,** bediscussieert de voorgestelde pathogene rol voor $\alpha E\beta7^+$ T-lymfocyten in IBD-patiënten. Onze onderzoeksgroep liet zien dat nieuw gediagnosticeerde IBD-patiënten statistisch significant lagere percentages van $\alpha E\beta7^+$ T-lymfocyten in hun ontstoken darm-biopten hebben dan gezonde volwassenen[4]. Bovendien lieten we zien dat patiënten ten tijden van endoscopisch inactieve ziekte hogere percentages $\alpha E\beta7^+$ T-lymfocyten in hun darmbiopten hadden, vergelijkbaar met de percentages van gezonde controles. Het zou daarom zelfs zo kunnen zijn dat $\alpha E\beta7^+$ T-lymfocyten nodig zijn om remissie te bereiken en/of te behouden in IBD-patiënten.

Een gedetailleerde review over dit onderwerp wordt gepresenteerd in **hoofdstuk 5b**. Naast de expressie van αEβ7 op pro-inflammatoire T-lymfocyten, wordt deze integrine ook teruggevonden op andere (regulatoire) immuuncellen (bijvoorbeeld regulatoire T-lymfocyten en dendritische cellen) en in andere organen dan de darm (bijvoorbeeld het brein, de longen, lever en nieren). Dit zou kunnen leiden tot ongewenste bijwerkingen in IBD-patiënten die behandeld worden met anti-β7 medicatie. De ontwikkeling van anti-β7 medicatie is gebaseerd op het idee dat IBD-patiënten een verhoogd aantal $\alpha E\beta7^+$ T-lymfocyten in hun ontstoken darm zouden hebben, wat een pathogene rol voor deze cellen zou kunnen impliceren. Echter, tot op heden is dit niet aangetoond. Integendeel, lagere percentages van $\alpha E\beta7^+$ T-lymfocyten worden gevonden in vergelijking met gezonde volwassenen. Toekomstig onderzoek op dit gebied zou zich moeten richten op het identificeren van de typen subsets en de functionaliteit van $\alpha E\beta7^+$ intestinale

T-lymfocyten in IBD-patiënten. Dit zou gedaan moeten worden door middel van functionele analyses waarbij ook TGF-β en retinolzuur worden meegenomen. Subsetanalyse moet worden uitgevoerd met andere markers zoals CD69, CD4/CD8 en markers van de uitrijping van T-lymfocyten. Daarnaast zouden ook αEβ7^{+}regulatoire T-lymfocyten en dendritische cellen in de darm onderzocht moeten worden. Dit onderzoek zou specifieke subgroepen van patiënten kunnen identificeren die wel behandeld kunnen worden met anti-β7 medicatie.

Serum biomarkers

Gemakkelijk beschikbare, minimaal invasieve, nauwkeurige markers van ziekteactiviteit bij IBD-patiënten die kunnen voorspellen hoe het ziektebeloop zal zijn of hoe de respons op een bepaalde behandeling zal zijn, zouden een substantiële bijdrage leveren aan vroege stratificatie van patiënten. In dit proefschrift onderzochten we verscheidene potentiële serummarkers in nieuw gediagnosticeerde, onbehandelde IBD-patiënten (**hoofdstuk 6 en 7**). Ons onderzoeksmodel bestond uit het serum van IBD-patiënten op het moment van diagnose en later in het beloop van hun ziekte, alsmede serum van gezonde volwassenen. Serologische antilichamen werden bestudeerd door middel van enzyme-linked immunosorbent assays (ELISA) en indirecte immunofluorescentie. Cytokines, chemokines, groeifactoren, oplosbare receptoren en andere mediatoren werden onderzocht door middel van een multiplex immunoassay.

Serologische antilichamen

Ondanks het feit dat er veel onderzoek is gedaan naar serologische antilichamen in volwassen IBD-patiënten, werd er in deze onderzoeken over het algemeen slechts op één tijdspunt een meting verricht, reeds nadat ziektecomplicaties waren opgetreden. In **hoofdstuk 6** bepaalden wij de serum antilichamen in nieuw gediagnosticeerde IBD-patiënten ten tijde van hun initiële diagnose en tevens op een tweede moment tijdens het beloop van hun ziekte. De combinatie van pANCA en ASCA zorgde voor een goed onderscheid tussen patiënten met CD en CU. Wij vonden geen associatie tussen de aanwezigheid van antilichamen op het moment van diagnose en verschillende ziekte-uitkomsten. In ons onderzoek, in patiënten in de dagelijkse klinische praktijk van een groot perifeer ziekenhuis, was de prevalentie van antilichamen relatief laag in vergelijking met de prevalentie in eerdere studies, uitgevoerd bij patiënten in academische ziekenhuizen[5,6]. De aanwezigheid van antilichamen op het moment van diagnose was gecorreleerd met de aan- of afwezigheid bij de tweede meting. De concentraties fluctueerden echter wel tussen deze twee meetmomenten, soms gerelateerd aan thiopurine-gebruik en behandeling met anti-TNF medicatie. Het zou daarom interessant zijn om deze antilichamen in een homogeen patiëntencohort te onderzoeken, voor en na het starten van eenzelfde medicijn (bijvoorbeeld infliximab, een anti-TNF medicijn), om de aan- of afwezigheid van de antilichamen te onderzoeken, alsmede de mogelijke veranderingen door de behandeling en de samenhang met de effectiviteit van de behandeling. De aan- of afwezigheid van pANCA in patiënten met CU

correleerde met ziekteactiviteit en zou mogelijk gebruikt kunnen worden bij het monitoren van ziekte, mede omdat het een sterke correlatie met de calprotectine waardes liet zien.

Toekomstig longitudinaal onderzoek van pANCA in patiënten met CU gedurende meerdere momenten tijdens hun ziekte, zowel tijdens actieve als tijdens inactieve ziekte, zou moeten laten zien of dit antilichaam als marker gebruikt zou kunnen worden. Als nieuwe serologische antilichamen getest worden in IBD-patiënten zou het goed zijn om dit in een cohort te doen uit de dagelijkse klinische praktijk, bij voorkeur vanaf het moment van diagnose, met longitudinaal vervolg en meerdere serumanalyses gedurende de ziekte.

Potentiële serummarkers

In **hoofdstuk 7** identificeerden we potentiële serummarkers voor CD door middel van een multiplex immunoassay in het serum van onbehandelde patiënten op moment van diagnose en wij vervolgden deze potentiële markers gedurende het ziektebeloop. Serumwaarden van sTNF-R2, sIL-2R en MMP-1 waren hoger in CD-patiënten dan in gezonde volwassenen, de waarden daalden significant ten opzichte van baselinewaarden ten tijde van periodes van remissie, terwijl ze stegen gedurende een opvlamming van de ziekte.

Nagenoeg alle pro-inflammatoire cytokines, welke verondersteld worden betrokken te zijn bij de pathogenese van IBD, waren ondetecteerbaar in het serum van onze patiëntengroep. Dit zou kunnen suggereren dat er een verplaatsing optreedt van deze cytokines van het bloed naar de ontstoken darm of dat er een verhoogde binding van deze cytokines aan oplosbare receptoren is.

Seriële metingen lieten een statistisch significante daling van nagenoeg alle potentiele serummarkers zien in patiënten die inactieve ziekte hadden gedurende de tweede meting. Dit suggereert dat er een associatie is tussen de waarden van de verschillende serummarkers en mucosale ziekteactiviteit. Bij de tweede meting was de combinatie van CCL-19, CXCL-13, sTNF-R1, en sIL-2R zeer accuraat in het identificeren van patiënten met actieve ziekte ten opzichte van patiënten met inactieve ziekte. De combinatie van deze markers zou dus mogelijk ziekte-opvlamming kunnen vaststellen zonder de noodzaak tot invasief endoscopisch onderzoek.

Lagere baselinewaarden van sTNF-R2 en VCAM waren geassocieerd met ziekte met veel opvlammingen terwijl een hogere baselinewaarde van sTNF-R2 was geassocieerd met een slechte respons op initiële behandeling met orale corticosteroïden.

Toekomstig onderzoek zou de voorspellende waarde van de bovenstaande potentiële serummarkers moeten bewijzen. In overeenstemming met onze bevindingen werd sIL-2R

recent voorgesteld als marker van mucosale genezing in IBD-patiënten [7]. Deze marker wordt al in de klinische praktijk gebruikt in andere patiëntencategorieën, bijvoorbeeld patiënten met sarcoïdose, en lijkt zodoende de meest aangewezen marker voor verder onderzoek in CD-patiënten. Verder onderzoek in IBD-patiënten naar potentiële markers waarbij simultaan gemeten wordt in zowel serum als in supernatant van de darm zou de relatie tussen serum- en darmwaarden van verschillende markers kunnen onthullen en daarmee meer inzicht kunnen geven in het etiologische mechanisme van de ziekte.

Onderzoek naar serummarkers in nieuw gediagnosticeerde patiënten met CU op moment van diagnose en gedurende het ziektebeloop zou ook in die patiëntengroep de potentiële markers openbaren. Daarnaast zou onderzoek naar serummarkers in homogene IBD-patiënten cohorten voor en na het starten van bepaalde medicamenten kunnen aantonen of het mogelijk is om de respons op een bepaalde behandeling te voorspellen.

Toekomstig onderzoek

In het algemeen onderstrepen onze bevindingen van verschillende immunologische profielen op het gebied van T-lymfocyteninfiltraat en serumanalyten, tussen patiënten met actieve endoscopische ziekte in vergelijking met gezonde volwassenen en patiënten met endoscopisch inactieve ziekte, de noodzaak tot het opsplitsen van deze subgroepen patiënten in al het komende onderzoek. Ondanks dat dit logisch klinkt, is het in de praktijk helaas nog niet gebruikelijk om dit onderscheid te maken en worden IBD-patiënten vaak vergeleken met gezonde volwassenen, onafhankelijk van de aan- of afwezigheid van actieve ziekte in de darm.

Synchroon aan de toegenomen incidentie van IBD, breidt het therapeutisch arsenaal snel uit, met vele nieuwe middelen die bijna op de markt komen. Dit zorgt voor kansen en uitdagingen voor artsen: hoe kiezen we het beste medicijn voor een specifieke patiënt? De huidige IBD-literatuur verplaatst de focus steeds meer van een symptoomgerichte behandelstrategie naar een zogenaamde targetgerichte behandelstrategie, waarbij de behandeling gestuurd wordt door objectieve criteria en door patiënt gerapporteerde uitkomstmaten, met als doel de ziektelast op lange termijn te verlagen [8,9]. Biomarkers zouden van substantieel belang zijn in het implementeren van deze targetgerichte behandelstrategieën op een gepersonaliseerde manier.

In dit proefschrift identificeerden we verscheidene potentiële biomarkers in IBD-patiënten en legden we hun aanwezigheid vast op verschillende momenten tijdens de ziekte. In toekomstig onderzoek zouden de best voorspellende markers gecombineerd moeten worden tot een online tool waarin het mogelijk is om het risicoprofiel van de individuele patiënt te matchen met de verschillende behandelopties om gepersonaliseerde geneeskunde te bereiken en betere ziekte-uitkomsten te krijgen. Om dit te bereiken is uitgebreid verder onderzoek nodig naar mogelijke biomarkers in homogene, goed gedefinieerde IBD-patiëntengroepen.

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ABBREVIATIONS

ACCA	anti-chitobioside carbohydrate antibodies
ALCA	anti-laminaribioside carbohydrate antibodies
AMCA	anti-mannobioside carbohydrate antibodies
ASCA	anti-*Saccharomyces cerevisiae* antibodies
AUC	area under the curve
CCR	chemokine receptor
CD	Crohn's disease
DC	dendritic cell
FACS	Fluorescence-activated cell sorting
GALT	gut associated lymphoid tissue
GZMA	granzyme A
HBI	Harvey-Bradshaw Index
HC	healthy controls
HEVs	high endothelial venules
IBD	inflammatory bowel diseases
IBDU	inflammatory bowel diseases type unclassified
ICAM-1	intercellular adhesion molecule 1
IEL	intraepithelial T lymphocyte
IFX	infliximab
ITGAE	gene expression of integrin alpha E
IQR	interquartile range
LFA-1	lymphocyte function-associated antigen 1
LPL	lamina propria T lymphocytes
LTα1β2	Lymphotoxin α1β2
mAb	monoclonal antibody
MAdCAM-1	mucosal adressin-cell adhesion molecule-1
MHC	major histocompatibility complex
MLN	mesenteric lymph node
MMPs	matrix metalloproteinases
pANCA	perinuclear anti-neutrophilic cytoplasmic antibodies
PE	phycoerythrin
PNAd	peripheral lymph node addressin
PPs	Peyer's patches
RA	retinoic acid
ROC	receiver operating characteristic
RTE	recent thymic emigrants
SES-CD	simple endoscopic score for Crohn's disease

SLOs	secondary lymphoid organs
STNFRs	soluble TNF receptor
TCR	T cell receptor
TGF-β	Transforming Growth Factor-β
TNF-α	tumour necrosis factor-α
TRECs	T cell receptor excision circles
Tregs	regulatory T cells
TCM	central memory T lymphocytes
TEM	effector memory T lymphocytes
TEMRA	effector memory T cells re-expressing CD45RA
TN	naive T lymphocytes
TRM	tissue-resident memory T cells
TLOs	tertiary lymphoid organs
UC	ulcerative colitis

9

Dankwoord
Curriculum vitae
List of publications

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CURRICULUM VITAE

Carolijn Smids werd op 24 februari 1988 geboren in Leiden en groeide op in Arnhem. In 2006 behaalde zij haar VWO-diploma aan het Lorentz Lyceum in Arnhem. Aansluitend startte zij met de studie verpleegkunde in Nijmegen. Na het behalen van haar propedeuse werd ze in 2007 alsnog ingeloot voor de studie geneeskunde aan de Radboud Universiteit in Nijmegen. In 2010 startte Carolijn met haar coschappen waarbij haar interesse in de Maag-, Darm- en Leverziekten werd gewekt tijdens haar seniorcoschap in het Rijnstate ziekenhuis te Arnhem. Haar wetenschappelijke stage maakte haar enthousiast voor de onderzoekswereld. In december 2013 begon zij in het Rijnstate ziekenhuis aan het promotieonderzoek getiteld "Profiling the immune response in early Inflammatory Bowel Disease" onder supervisie van dr. M.J.M. Groenen, dr. E.G. van Lochem, dr. P.J. Wahab en prof. dr. J.P.H. Drenth In maart 2017 begon Carolijn met de opleiding tot Maag-Darm en Leverarts in het Rijnstate te Arnhem bij opleider dr. L.J.M. Reichert (Interne Geneeskunde) en opleider dr. M.J.M Groenen (Maag-, Darm- en Leverziekten). Carolijn woont samen met Maarten Besjes en hun dochter Juliët in Elst.

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